8-2017

Porphyromonas gingivalis infection in gestational diabetes mellitus and survival in tobacco smokers.

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PORPHYROMONAS GINGIVALIS INFECTION IN GESTATIONAL DIABETES MELLITUS AND SURVIVAL IN TOBACCO SMOKERS

By

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A Dissertation

Submitted to the Faculty of the

School of Interdisciplinary and Graduate Studies

In Partial Fulfillment of the Requirement for the Degree of

Doctor of Philosophy in Interdisciplinary Studies

School of Interdisciplinary and Graduate Studies

University of Louisville,

Louisville, KY

August 2017

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ACKNOWLEDGEMENTS

Foremost, I would like to express my sincere gratitude to my mentor, Dr. David A Scott for his continuous support and encouragement. I remain indebted to him for his patience and the valuable research experience I gained during my training in his lab.

I would also like to thank my committee members Dr. Richard J. Lamont, Dr. Jan S. Potempa, and Dr. Deborah R. Yoder-Himes for their valuable suggestions. Special thanks to Justin, Anna, and Diane for teaching me every single procedure with great patience. I would also like to extend my thanks to the Dr. Lamont, Dr. Potempa, Dr. Demuth and Dr. Wang labs, for the equipment. I would also like to thank my lab members Gwyneth, Zhen, Shilpa, Atul, and Rishi for their constant support in the lab.

Many thanks to my parents Mr. Jagan Mohan Rao and Mrs.Uma Rani, my brother Sarad Sai, my grandmother Anasuya Devi and my uncle Mr. Ranga Rao for their unconditional love, motivation and financial support. I would also like to thank my friends, my extended family Manju, Madhuri Collooru, Bharath Sreekantam, Srikanth Peetha, Swathi, Lahari, Satya Upadhyayula, Srikanth Ravipati, TJ, Gowthami, Dalina and Laxmi garu for their emotional support and for always being there for me.

ABSTRACT

PORPHYROMONAS GINGIVALIS INFECTION IN GESTATIONAL DIABETES MELLITUS AND SURVIVAL IN TOBACCO SMOKERS

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July 28, 2017

This thesis contains a clinical project and distinct basic science project.

Gestational diabetes mellitus occurs in 4% of pregnancies and increases the risk of birth defects, pre-term birth, and miscarriage. Gingivitis during pregnancy also increases the risk for poor pregnancy outcome. Gingivitis is a bacterial-induced disease, and specific plaque pathogens have been associated with systemic sequelae to periodontal inflammation. Therefore, we set out to monitor oral infection with three key periodontopathogens (*Porphyromonas gingivalis*, *Filifactor alocis,* and *Treponema denticola*) and the systemic inflammatory burden [C-reactive protein (CRP)] in pregnant women with and without gingivitis and gestational diabetes. Gingivitis during pregnancy leads to a dramatic increase in systemic CRP (mean 8116 vs. 2495 ng/ml, $p < 0.01$), as determined by Enzyme Immunoassay (EIA). As expected, gingivitis during pregnancy was associated with oral infection with *P. gingivalis*, *F. alocis* and *T. denticola* and combinations thereof (all $p < 0.01$), as determined by salivary PCR. Gestational diabetes mellitus was also associated with increased infection with individual and multiple periodontopathogens, including *P. gingivalis*. Thus, diabetes and gingivitis act in concert to increase risk biomarkers for poor pregnancy outcome. Actual pregnancy outcomes in the study population are currently being monitored.

The majority of cases of chronic periodontitis in developed nations are tobacco-related. For periodontal pathogens, such as *P. gingivalis*, to induce or exacerbate periodontal diseases in smokers, they must first be able to survive the highly complex composite toxic insult represented by cigarette smoke. While it is clear that *P. gingivalis* is resistant to highdoses of cigarette smoke and tobacco constituents, the survival mechanisms are entirely unknown. Therefore, we first generated a *P. gingivalis* ATCC 33277 transposon sequencing (Tn-Seq, Library 1) and determined the putative minimal essential genome for *in vitro* growth in complex media in conjunction with a separate *P. gingivalis* ATCC 33277 Tn-Seq library generated by *Klein et al.* (Library 2). In all, 281 genes (61%) identified by Library 1 were common to Library 2. Many of these common genes are involved in crucial metabolic pathways, notably pyrimidine cycling as well as lipopolysaccharide, peptidoglycan, pantothenate and coenzyme A biosynthesis, and nicotinate and nicotinamide metabolism. Also in common are genes encoding heat-shock protein homologs, sigma factors, enzymes with proteolytic activity, and the majority of Sec-related protein export genes. In addition to facilitating a better understanding of critical physiological processes, transposon-sequencing technology has the potential to identify novel strategies for the control of *P. gingivalis* infections. Those genes defined as essential by two independently generated TnSeq mutant libraries are likely to represent particularly attractive therapeutic targets.

Next, we tested Library 1 for genes essential for the survival of exposure to cigarette smoke extract. Of the 2155 *P. gingivalis* ATTCC 33277 genes, 257 (8%) were found to be essential for growth upon cigarette smoke exposure. The essential genes were distributed throughout the bacterial genome. Genes with products that function in DNA metabolism, energy metabolism, signal transduction, protein fate and transport and binding proteins were best represented within the essential gene set. In a competition assay of the mutants

of genes of biological interest, the following mutants were outcompeted by the wild type, *P. gingivalis* ATCC 33277 strain: PGN_1524 encoding uncharacterized protein, PGN_1474 (*luxS*) encoding S-ribosyl homocysteine lyase, PGN_1200 encoding putative ATPase,PGN_1444 (*carA*) encoding carbamoyl- phosphate synthase small chain, PGN_0770 (*rnz*) encoding ribonuclease Z, PGN_0714 encoding probable pyrazinamidase/ nicotinamidase, PGN_0491 encoding probable phosphotyrosine protein phosphatase , PGN_0287 (*mfa1*) encoding minor fimbrium subunit Mfa1 , PGN_0088 encoding putative transcriptional regulator, and PGN_0388 (*tpx*) encoding putative thiol peroxidase. We confirmed the respective gene fitness in a Gifu anaerobic media conditioned with cigarette smoke extract (GAM-CSE) model. Further competition assays of the top 10 genes in the identified gene list, according to the fold change and complementation assays, could provide some of the first insights into how cigarette smoke changes the *P. gingivalis* phenotype in a manner likely to promote colonization and infection. Those genes identified as conditionally essential are likely to represent attractive therapeutic targets to combat *P. gingivalis* infection in smokers.

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CHAPTER 1

INTRODUCTION

Next to dental caries, periodontal diseases are the most prevalent oral diseases in the world, affecting 10-15% of adult population, according to the WHO [1]. According to the CDC report, 47.2% of adults in the United States aged 30 years and older have some form of periodontal disease [2]. Periodontal diseases refers to inflammation of the periodontium, the supporting tissues of the tooth, and are the leading causes of tooth loss [3]. Clinically, periodontal diseases are characterized by gingival inflammation, gingival bleeding, and increased periodontal pocket depth [4]. According to the American Academy of Periodontology, periodontal diseases are broadly classified as gingivitis and periodontitis [5]. Gingivitis is the inflammation of gingiva without any bone loss, usually associated with the presence of bacteria. Periodontitis is defined as inflammation of the gingiva extending into the adjacent attachment apparatus of the tooth [6]. Chronic periodontitis is the most prevalent form of periodontitis and has been associated with increased prevalence of diabetes mellitus, AIDS, leukemia, down's syndrome, cardiovascular diseases, rheumatoid arthritis and pre-term low birth weight babies [7-11]. Chronic periodontitis is multifactorial, and its pathogenesis is characterized by the interaction of host (genetics), agent (oral biofilm), and environmental (patient behaviors like smoking, medications or systemic diseases) [3]. The pre-requisite for the development of the periodontal diseases is the presence of pathogenic biofilms [12].

1.1. Oral biofilm:

Oral biofilm is defined by Costerton *et al.*, as a structured community of bacterial cells enclosed in self-produced polymeric matrix and adherent to an inert or living surface. The first step in the oral biofilm formation process is the formation of the salivary pellicle, a thin

acellular film that forms on any surface upon exposure to saliva, providing a niche for the colonization of microorganisms through autogenic succession. Further oral bacteria secrete mucin, albumin, glycoproteins, acidic proline-rich proteins, etc. which form the matrix of the biofilm [13]. The microbes that colonize by adhering to salivary pellicle and initiate biofilm formation are primary colonizers. Primary colonizers are mainly commensals of the oral cavity such as Gram-positive bacilli and cocci including *Streptococcus sanguis*, *Streptococcus oralis*, *Streptococcus mitis* and *Actinomyces viscosus*. If left undisturbed, the primary colonizers promote the colonization of more pathogenic late colonizers such as *Treponema denticola*, *Porphyromonas gingivalis* and *Tannerella forsythia* [14, 15]. The biofilm is formed through a coordinated process, supporting a broad range of bacteria by providing a favorable niche for the periodontal pathogens [16]. The presence of certain species like *P. gingivalis* leads to dysbiosis, resulting in periodontal destruction [17]. However, the presence of biofilm alone is not sufficient to cause periodontal diseases [18]. Host immune response to these pathogens plays a crucial role in the periodontal tissue destruction.

According to the polymicrobial synergy and dysbiosis (PSD) model, oral dysbiosis plays a crucial role in the pathogenesis of periodontal diseases. *P. gingivalis,* which is considered a keystone pathogen, alone failed to induce bone loss in germ-free mice but was able to induce bone loss when combined with other oral commensals [19]. *P. gingivalis* plays a crucial role in the pathogenesis of periodontitis by altering the oral biofilm composition and the host immune response to these altered biofilms [20, 21]. As per PSD model, periodontitis is believed to be initiated by synergistic and dysbiotic microbial communities [21]. Dysbiosis is characterized by the relative abundance of pathobionts, commensals of the biofilm that under certain conditions can cause pathology [22, 23], compared to commensals in the biofilm. The results of human clinical studies are in agreement with the

observations identified in animal models regarding the PSD model. Clinically, it has been shown that in the case of periodontal diseases, there is a decrease in the commensal organism *Streptococcus sanguis* [24, 25]. The results of transcriptional profiling of microbial samples from periodontally healthy versus diseased sites have demonstrated that the majority of potential virulence factors upregulated in human disease are derived from the bacteria associated with periodontal health [26]. Many recent studies, using Human Microbiome Project data set as a reference, have shown the difference in the biofilm structure and its functional capacities [27-30]. Studies involving recent advancements like 16S rRNA and shotgun sequencing have also confirmed the existence of significant differences in microbial communities between healthy and periodontal disease individuals [28-31]. This finding is further confirmed by the case-control study performed using Illumina Miseq Desktop Sequencing of subgingival samples [27, 32]. Cluster analysis by principal component analysis showed that the bacterial communities were different in healthy and periodontally diseased groups; *Porphyromonas*, *Treponema*, and *Filifactor* species were significantly enriched in the diseased group. Also, it has been shown that the chronic periodontitis sites contained greater microbial diversity and species richness compared to healthy control sites [27, 32].

In an oral biofilm, oral microorganisms interact through inter-microbial adhesion, cell signaling using cell-to-cell contact. Metabolic communications, including quorum sensing, also play a major role in maintaining the integrity of oral biofilm and an essential role in microorganism survival [33, 34], leading to coordinated gene expression within the microbial community that in turn influences the pathogen's ability to cause disease [35, 36]. Metatranscriptome analysis studies showed that there to be overexpression of biological functions related to flagellar motility, peptide transport, iron acquisition, beta-lactam degradation and the biosynthesis of the lipid A component of endotoxins in the

dental biofilms collected from active periodontal sites [26, 37]. Thus, in an individual with periodontitis, there is altered expression of genes related to particular physiological processes that help the microbes to survive in the changed environmental conditions of the dysbiotic biofilm . Further, these dysbiotic biofilms can lead to chronic inflammation by stimulating the host inflammatory response [21, 38].

In summary, periodontal diseases may be triggered by a conglomeration of several bacterial species, rather than a single pathogen, and be dependent on the microbial communities and their interactions. Dysbiosis of oral biofilms plays a crucial role in the pathogenesis of periodontal diseases by aggravating the host immune response to oral biofilms, leading to inflammation and bone loss [39]. Local environmental changes such as increased gingival inflammation, bleeding on probing, and increased periodontal pocket depth periodontal also alter bacterial gene expression related to survival fitness of the microbes in the biofilms. While most organisms in the subgingival microbiota are considered commensals, several bacteria have been implicated as periodontal pathogens including *Porphyromonas*, *Treponema,* and *Filifactor*. However, *P. gingivalis* and *T. denticola* were found in both healthy and periodontally diseased individuals. In this study, we are interested in studying *P. gingivalis*, *T. denticola, and F. alocis* as they have shown a strong association with periodontal diseases.

1.2. *Treponema denticola*:

T. denticola is a Gram-negative bacterium from the Spirochetes family. It is motile, slender, helically-shaped, and flexible. It has been routinely isolated from human subgingival plaque and is strongly associated with changes in the subgingival ecology characteristic of disease sites [40-42]. *T. denticola* is considered as one of the main etiological agents for periodontitis [41, 43]. In contrast to the wide diversity of phylotypes in healthy sites, only a few phylotypes are found in diseased sites [44]. *T. denticola* can

lyse and damage periodontal cells, adhere to epithelial cells and fibroblasts, co-aggregate to biofilm-bridging organisms, and can produce proteins and polypeptides that interact with the host immune response [40]. *In vitro* studies have shown that components of *T. denticola* can induce a range of pro-inflammatory cytokines such as interleukin 1(IL-1), tumor necrosis factor (TNF), IL-6 and IL-8 [45]. It has been found that the proportion of *T. denticola* increases significantly in periodontal disease biofilms. *T. denticola* is typically detected together with other pathogens *P. gingivalis* and *T. forsythia* [46]. In murine models of periodontitis, synergy between organisms has been demonstrated by inoculation of animals with *P. gingivalis* together with either *T. denticola* or *T. forsythia,* resulting in more extensive alveolar bone destruction than inoculation with a single species [47, 48]. Thus, the prevalence of *T. denticola* in periodontal pockets, together with high numbers of other proteolytic Gram-negative bacteria, may play a major role in the progression of periodontal disease. One of the challenges of researching treponemes, such as *T. denticola*, is the difficulty of their cultivation *in vitro*. *T. denticola* requires the presence of fibronectin coating to facilitate its attachment, and it is difficult to obtain a model system for biofilm growth for pure cultures of *T. denticola* [49]. Hence little work has been done on *T. denticola* biofilm growth.

1.3. *Filifactor alocis:*

Recent advances in sequencing techniques have allowed the identification of microorganisms within the subgingival dental biofilm, which had previously been unrecognized because of culture difficulties. *F. alocis* has been identified as potential pathogen in recent non-culture based studies [27, 50]. *F. alocis* is a fastidious, Gram-positive, obligately anaerobic rod. It is considered to be an important pathogen in the formation of subgingival biofilm and the onset of periodontal diseases [31]. Owing to its high prevalence and abundance in periodontal disease sites compared with healthy sites,

it is considered as a potential marker for active disease [27, 51, 52]. *F. alocis* is relatively resistant to oxidative stress and possesses several virulence factors, such as sialoglycoproteins, proteases, adhesion molecules, neutrophil-activating protein A, and a calcium-binding acid repeat protein [31]. *F. alocis* can stimulate the production of host pro-inflammatory cytokines IL-1β, IL-6, and TNF, which in turn can stimulate pathways that activate osteoclasts and increase alveolar bone resorption [53, 54]. *F. alocis* can also interact with other oral bacteria and form biofilms [55]. *F. alocis* has also been shown to enhance the virulence or the growth of other pathogens, promoting the pathogenesis of periodontitis [31]. For example, *F. alocis* synergistically interacts with *P. gingivalis* [56], enhancing biofilm formation and invasion of epithelial cells by when in coculture. *F. alocis* also interacts synergistically with *F. nucleatum in vitro* [57]. It was previously shown that *F. alocis* is involved in numerous spatial arrangements with other organisms, indicating its role as one of the architects of structural organization within periodontal biofilms [55].

1.4. *Porphyromonas gingivalis:*

P. gingivalis is a Gram-negative, black-pigmented, obligately anaerobic organism, and the most studied of the periodontal pathogens. This species was found in 85.75% of subgingival plaque samples from patients with chronic periodontitis [58]. Colonization by *P. gingivalis* is also associated with some systemic diseases, including cardiovascular diseases, rheumatoid arthritis, and Alzheimer's disease [59-61]. Its occurrence is higher in subjects with chronic periodontitis compared to periodontally healthy subjects [62, 63].

P. gingivalis is considered to be a keystone pathogen in the pathogenesis of periodontitis, due to its disproportionate influence on the microbiome in relation to its abundance [17, 64]. *P. gingivalis* was identified as a keystone pathogen in a study using a combination of culture-based methods and next-generation sequencing(NGS) [65]. Human studies have also shown that *P. gingivalis* is present in low abundance when compared with the total

oral microbiota in disease sites [52]. *P. gingivalis* benefits from a variety of interactions with other bacteria under deprived oxygen conditions. Studies have also demonstrated that *P. gingivalis* can be present in the subgingival biofilm and colonize the epithelium in healthy individuals [66, 67]. *In vivo* studies have shown that even though *P. gingivalis* is present in low numbers, its presence enhances the growth of pathogenic microflora which in turn leads to periodontal diseases [19]. On its own, *P. gingivalis* does not induce disease in germ-free mice, yet it can shift the balance of microflora in oral cavity towards a more pathogenic community, resulting in periodontal diseases. Thus pathogenicity of *P. gingivalis* is related not only to its physiological state but also to its interactions with other oral microflora [22].

The means by which early colonizers facilitate colonization by *P. gingivalis* include the provision of attachment sites for adherence, the supply of growth substrates, and reduction of oxygen tension to the low levels required for growth and survival [68]. *P. gingivalis* can adhere to both early and late colonizers of oral biofilm. *P. gingivalis* plays a crucial role in the autogenic succession of oral microflora towards more Gram-negative bacteria leading to dysbiosis [28, 62, 69]. In a mouse model, it has been shown that, despite low numbers, *P. gingivalis* created dysbiotic changes in the oral biofilm. Further, it has been shown that dysbiosis can be triggered by keystone pathogens such as *P. gingivalis* and is characterized by an increase in the prevalence of pathobionts [24, 25, 70]. A recent study also proved the concept of keystone pathogen-mediated (*P. gingivalis*) PSD model to explain the etiology of periodontitis [71].

In vitro studies revealed that *P. gingivalis* produces a wide array of putative virulence factors including fimbriae, capsules, lipopolysaccharide (LPS), lipoteichoic acids, hemagglutinins, gingipains (secreted proteases), outer membrane proteins, and outer membrane vesicles [72, 73]. However, the mere presence of virulence factors itself is not

sufficient to induce attachment and bone loss. Host immune response to these virulence factors, and to the bacteria as a whole, plays a crucial role in the pathogenesis of periodontitis [74, 75]. The virulence factors of *P. gingivalis* are known to suppress the host immune response, thus promoting the growth of commensals in the oral biofilm [39]. *In vitro* studies have shown that *P. gingivalis* can induce proinflammatory cytokines such as IL-1α, IL-1β, TNF, IL-6, and IL-8 [76, 77]. A study by Guentsch *et al.*, showed that extracellular release of reactive oxygen species (ROS) by neutrophils occurred when exposed to *P. gingivalis*. This phenomenon may lead to destruction of the bacteria and damage to the surrounding periodontal tissue. *P. gingivalis* can also upregulate the collagen-degrading ability of some human gingival fibroblast (HGF) cell lines [78], thus may have a role in enhancing periodontal tissue degradation *in vivo*.

1.5. *P. gingivalis* virulence factors:

Fimbriae

The fimbriae of *P. gingivalis* are thin, filamentous, cell surface protrusions. Two classes of fimbriae exist, Type I (major) fimbriae and type II (minor) fimbriae. Type I (major) fimbriae have significant roles in colonization and invasion, whereas type II (minor) fimbriae possess a higher pro-inflammatory capacity [79-81]. Fimbrillin, a protein in the major fimbriae, is encoded by *fimA* gene. This gene was classified into six genotypes according to its genomic differences [82, 83]. *fimA* type I was associated with periodontal health. Genotype II has greater adhesive and invasive properties, has been observed to be more prevalent in periodontal patients, and is associated with more aggressive forms of periodontal disease. In mouse models, type I and III induced weak inflammation whereas type II, Ib, IV, and V induced strong inflammation [84]. In experimental periodontitis models, fimbriae induced bone destruction. In rat models, infection with non-fimbriated *P. gingivalis* strains exhibited reduced periodontal bone loss, compared with

infection with fimbriated strains [85]. Immunization against *P. gingivalis* fimbriae protected against bone loss in gnotobiotic rats [86]. Other properties of both major and minor fimbriae include the induction of pro-inflammatory cytokines and production of matrix metalloproteinases (MMPs), such as IL-1, IL-6, IL-8, TNF, and MMP-9, by various host cells [87-89]. *P. gingivalis* fimbriae can signal through either TLR2 or TLR4. Activation of TLR2 by fimbriae results in a distinctive signaling pattern compared with activation by *P. gingivalis* LPS [90]. The differential signaling pattern, in turn, activates the binding capacity of CR3 and allows for internalization of *P. gingivalis* in macrophages and reduction of IL-12 production, which may collectively inhibit bacterial clearance [91].

Proteases

Proteases were classified as serine, cysteine, and metalloproteinase depending on the catalytic mechanisms. These include gingipains (Arg- or Lys-), periodontain (cysteine endopeptidase), PrtT proteinase, Tpr proteinase, collagenase (prtC gene), prolyl tripeptidyl peptidase (serine exopeptidase), dipeptidyl-peptidase IV (serine exopeptidase known as glycylprolyl peptidase, a product of the dppIV gene), dipeptidyl-peptidase VI (putative cysteine exopeptidase), amino-peptidase P, oligo-peptidase O and gelatinase (a proteinase degrading type IV collagen, gelatin, low-molecular-mass-kininogen and transferrin) [92]. Of these, the collagenases, aminopeptidases, and the trypsin-like proteases are critical to *P. gingivalis* pathogenesis [72].

P. gingivalis, an asaccharolytic organism, expresses a set of trypsin-like proteases that degrade proteins to use as an energy source, for processing of *P. gingivalis* proteins such as fimbriae and also potentiates inflammation [93, 94]. Gingipains are cysteine proteases that can cleave the proteins at arginine and lysine specific sites [95]. A variation on the genotype level of the proteolytic enzymes (gingipains) has been reported [96, 97]. *P. gingivalis* produces Lys-X and Arg-X peptide bonds specific proteases (Kgp, RgpA, and

RgpB gingipains) which are considered as a major virulence determinant. The activation of inflammation is part of a basic survival strategy, increasing the protein-rich gingival crevicular fluid bathing the gingival sulcus while dampening killing mechanisms in phagocytic cells [98]. Three types (type A, B, and C) of the Arg-specific cysteine proteinase-encoding genes (*prpR1/rgpA*) and two (type I and II) of the Lys-specific cysteine proteinase-encoding genes (*kgp*) were identified in these studies. These gingipains can up-regulate MMP-8 and MMP-3 expression in rat mucosal epithelial cells and activate latent MMPs [99, 100]. MMPs activation may, in turn, induce connective tissue and bone destruction leading to periodontitis [74]. Gingipains also deregulate the various components of the immune response [101] which, in turn, hampers bacterial clearance and facilitates bacterial invasion.

Capsule

The capsule is another important virulence factor of *P. gingivalis* [102, 103]. Six different serotypes of capsules have been identified depending on capsular polysaccharide K-antigens and their capacity to generate systemic IgG antibodies [104]. Bacterial encapsulation is positively correlated with increased resistance to phagocytosis [105]. In comparison to non-encapsulated strains, encapsulated *P. gingivalis* strains are shown to be highly invasive, causing spreading infection in a murine lesion model and more resistance to phagocytosis by PMN [106, 107]. *P. gingivalis* capsule polysaccharide (CPS) mutant strain was found to be a more potent inducer of cytokine synthesis by human gingival fibroblasts compared with the corresponding wild-type strain. Thus CPS hampers the innate immune response [102, 103]. In an animal model, immunization with *P. gingivalis* capsule resulted in a decrease in alveolar bone loss induced by *P. gingivalis* [108].

Lipopolysaccharide (LPS)

LPS is a cell wall component of Gram-negative bacteria and is composed of O-antigen, core polysaccharide and lipid A. Lipid A exists predominantly in two forms in *P. gingivalis* - the tetra-acylated and penta-acylated forms. The penta-acylated lipid A activates TLR4, whereas tetra-acylated lipid A acts as a TLR4 antagonist [109, 110]. *P. gingivalis* LPS is thus a weaker cytokine stimulator compared with that of other Gram-negative bacteria. *In vitro*, it stimulates the production of pro-inflammatory cytokines such as IL-1α, IL-1β, IL-6, IL-8, IL-18 and TNF in monocytes [75, 111-113]. In animal models, it has been shown that *P. gingivalis* LPS triggers pro-inflammatory responses and bone resorption in the oral cavity [114]. In summary, *P. gingivalis* LPS leads to host immunological deregulation, thus facilitating the *P. gingivalis* adaptation and survival.

Recent studies showed that the expression of virulence factors of *P. gingivalis* is regulated in response to environmental changes. To ensure optimal nutrient uptake in response to environmental changes, *P. gingivalis* adopts various methods to utilize iron and heme [115]. It was found that *fimA* gene expression was decreased by approximately 50% in response to hemin limitation and the presence of serum or saliva in the growth medium [116]. Environmental conditions influence the changes in *P. gingivalis* lipid A acylation. Penta-acylated lipid A is converted into tetra-acylated lipid A when there is inflammation (high hemin availability), thus modulating host immune response [117]. Thus, *P. gingivalis* adapts to the environmental changes in the oral cavity for its survival within the host.

1.6. Host influence on *P. gingivalis*

Clinically periodontal diseases are characterized by gingival inflammation, bleeding on probing and increased pocket depth. Apart from the host immune response, bacterial adaptation to these local host environmental changes also plays a significant role in

survival and towards the pathogenesis of periodontal diseases [118]. It has been shown in many studies that *P. gingivalis* can alter expression of several genes in response to varied environmental conditions as a part of its survival strategy.

In response to a change in pH or temperature, *P. gingivalis* alters the biophysical properties of its membrane lipids [22]. Despite being anaerobe, *P. gingivalis* is micro-aerotolerant. It has been shown that on exposure to oxygen, *P. gingivalis* adapts to the aerobic conditions through upregulation of genes encoding adhesins and phosphatases, and the downregulation of protease-encoding genes and proteins interfering with adhesion [119]. On exposure to atmospheric oxygen, there is upregulation of genes involved in oxidative stress, and in virulence factors involved in heme acquisition, presumably as a protective mechanism against the molecular oxygen and the reactive oxygen species [120]. Heme is an essential nutrient for *P. gingivalis* survival. In a combined quantitative proteomic and transcriptomic analysis study, it has been shown that *P. gingivalis* upregulates the production of several proteins linked to metabolism, oxidative stress response, virulence, and invasion of host cells to cope with heme limitation [121]. *In vivo* studies have shown that the expression of virulence factors in *P. gingivalis* increase after hemin limitation [121, 122]. The heme-binding lipoprotein HmuY, GAPDH and some of the cell-surface-located C-terminal domain (CTD) family proteins, including RgpA, HagA, CPG70, and PG99, are increased in biofilm cells. Concurrently, the heme-binding protein IhtB, TonB-dependent receptor P92, Kgp, lipoprotein RagB, endopeptidase PepO, a glutamine cyclotransferase-related protein, fumarate reductases FrdAB, and ribosomal proteins L10, L13 and L25 are significantly decreased. There is a differential response of *P. gingivalis* to varying concentrations and durations of hydrogen peroxide [123]. As a response to shorter exposure to H_2O_2-induced oxidative stress, genes involved in DNA replication, recombination and repair were upregulated [123, 124]. After

a longer exposure, several genes involved in protein folding/repair were upregulated whereas those involved in translation were downregulated. Thus, *P. gingivalis* is capable of sensing and rapidly responding to changes in its environment.

1.7. *P. gingivalis* strains:

Different clones of *P. gingivalis* in subgingival plaque samples have been found [125, 126]. In "Java Project on Periodontal Disease", a longitudinal study performed over a span of 8 years to investigate the clonal stability of *P. gingivalis* [127], 24 *P. gingivalis* genotypes were detected that occurred in one subject only. This heterogenicity among *P. gingivalis* strains might arise due to mobile genetic elements such as many insertional sequences (IS) and transposons; pathogenic islands like the *rag* locus [128-130]. Genetic variation also arises as a consequence of conjugal transfer leading to allelic exchange in *P. gingivalis* [131-133].

The *rag* locus, a novel pathogenicity island, encodes RagA and RagB, entities that constitute a membrane transport system. RagA is an 115-kDa TonB-dependent outer membrane receptor, and RagB is a 55-kDa lipoprotein constituting an immunodominant outer antigen [134]. Several studies suggested the virulence of *rag* locus. In a study conducted in Columbia, 32 *P. gingivalis* isolates were analyzed and shown to contain a greater bacterial diversity due to the *rag* locus. Four *rag* locus *P. gingivalis* variants differ in their pathogenicity [134-136]. *P. gingivalis* carrying *rag*-1, *rag*-3 was more predominant in chronic periodontitis patients in Northeast China [137]. *P. gingivalis*- *rag* locus gene mutants show reduced virulence in a mouse model study [138].

P. gingivalis strains are classified as invasive or non-invasive strains based on differences in the ability to cause soft-tissue abscesses following subcutaneous injections at distant or local sites [139]. Different strains were also identified depending on their capacity to

cause periodontal bone loss, serum and saliva antibody expression and death in mice [140-142]. A difference in both alveolar bone resorption and the systemic acquired immune response was also found among different strains of *P. gingivalis* [143, 144].

In summary, several strains of *P. gingivalis* have been identified. Chronic periodontitis is not associated with a particularly virulent clonal type [145]. ATCC 33277 is one of the most commonly employed strains in periodontal research. It is pathogenic in experimental animals following oral infection [146]. Thus, we used *P. gingivalis* ATCC 33277 strain in our study in chapter 3.

1.8. *P. gingivalis* ATCC 33277 genome:

Naito *et al.* presented the whole genome sequence of *P. gingivalis* ATCC 33277[147]. The genome of ATCC 33277 comprised a single circular chromosome of 2,354,886 bp with an average G + C content of 48.4% [147]. The ATCC 33277 genome contained 2155 genes, 2090 protein coding sequences (CDSs) with a mean size of 970 bp, covering 86.1% of the whole chromosome sequence, approximately 117,000 TA sites, and no plasmids [32, 147, 148]. It contains 4 RNA operons and 53 tRNA genes that provide specificity for amino acids. ATCC 33277 contained a variety of mobile genetic elements [147]. Only four NCBI annotated genes coding hypothetical proteins lack TA sites [148]. A total of 93 insertion sequence elements and 48 miniature inverted-repeat transposable elements were found in ATCC 33277. Insertion sequences are the simplest transposable elements, encoding a transposase and can be as short as 600–700 bp. The insertion sequence elements identified in ATCC 33277 were classified into six types, ISPg1–ISPg6, all of which are also present in W83 [149].

Apart from ATCC 33277, the complete genome sequence of the strains W83, HG66, TDC60, and JCVISC001 are known. Three copies of 16S rRNA sequences in HG66 are

identical to the four copies of 16S rRNA sequences in ATCC 33277, suggesting a close evolutionary lineage between HG66 and ATCC 33277. Genome-wide comparisons based on Rapid Annotation using Subsystem Technology (RAST) also showed that for the overall biological functions of the genomes, ATCC 33277 is closer to HG66 compared to other strains. By comparing genome sequences of ATCC 33277 and W83 strains, 461 ATCC 33277-specific and 415 W83-specific predicted protein coding sequences were identified. Between the two strains, 175 regions with genomic rearrangements were observed. These rearrangements occurred as a result of various mobile genetic elements such as insertion sequences, miniature inverted-repeat transposable elements, transposons, and conjugative transposons (CTns) [147].

In summary, the expression of virulence factors by the periodontal pathogen is often regulated in response to changes in the external environment. In the subgingival area, bacteria experience dramatic environmental changes resulting from host eating and oral hygiene patterns, gingival crevicular flow rate variability, and the degree of bleeding. In response to these dynamic processes, bacteria often regulate gene expression to maintain optimal phenotypic properties. Many studies have shown that each virulence factor plays important roles to hamper the cell-mediated immune response in the host. However, in the host, the bacteria express a whole subset of virulence factors rather than any single virulence factor. Thus, analysis of the bacterial genome is crucial to understand the properties and role of virulence factors as a whole. In chapter 2, the generation of a *P. gingivalis* ATCC 33277 transposon mutant library is described. We compared the generated library with the TnSeq library generated by Klein *et al.*, to identify the common inherently essential genes in *P. gingivalis* ATCC 332777 strain. Further, the generated mutant library can be tested under various experimental conditions to investigate the conditional genes essential for *P. gingivalis* survival in those conditions.

In a susceptible host, dysbiosis can trigger abnormal host responses leading to the destruction of periodontal tissues and periodontal diseases [6, 150, 151]. The host-bacterial interactions are estimated to account for almost 80% of the risk of periodontal tissue damage [152]. A potentially virulent microbe remains avirulent in an insusceptible host. Thus, the presence of variable complex microbial patterns in a susceptible host and the inflammatory immune response plays a significant role in the outcome of periodontal diseases. The result of this interaction is, in turn, dependent on many other risk factors including smoking, systemic conditions such as gestational diabetes mellitus (GDM), and other factors including age, gender, and socioeconomic status.

1.9. Periodontal diseases and tobacco smoking:

Cigarette smoking is strongly associated with multiple diseases, including lung cancer, chronic obstructive pulmonary disease, heart disease, respiratory infection, and periodontitis [153-156]. It is considered to be the single major preventable environmental risk factor for chronic periodontitis through its influence on the microbial composition of the biofilm [28, 62, 157, 158] and the host immune system [155].

Smoking-related periodontitis is a significant global public issue, as there are >1 billion adult smokers worldwide. Approximately half of the periodontitis cases in the U.S.A. could be attributable to smoking, and current smokers were about four times more likely to be diagnosed with periodontitis than never smokers [159]. Smokers are refractory to periodontal treatment compared to non-smokers [160]. Cross-sectional studies have shown that smokers are two to seven times more likely to present with periodontitis, compared to non-smokers [159, 161]. Clinical studies have demonstrated that smokers have the more severe periodontal disease [162-164]. A recent country wide epidemiological study conducted in Sweden suggested a strong association between smoking and moderate and severe alveolar bone loss overall [165]. In a cohort study

carried out in New Zealand, it has been found that the extent and prevalence of alveolar bone loss was greater in smokers compared to never smokers and former smokers [166]. In a large population-based prospective cohort study of men and women in the state of Brandenburg, Germany, a strong dose-dependent association between cigarette smoking and the prevalence and incidence of tooth loss has been found. This association is independent of other risk factors and potential confounders [167]. In studies, conducted using cloning and sequencing methods, smokers are associated with higher prevalence of periodontal pathogens compared to non-smokers [168]. In a Dunedin birth cohort study, investigators followed up people born in 1973. They confirmed the strong association between chronic smoking and periodontal disease [169].

Tobacco smoke contains over 7000 different chemicals, including carbon monoxide, hydrogen cyanide, reactive oxidizing radical. Sixty of these chemicals are known or suspected carcinogens [170] and the most abundant alkaloid is nicotine . Tobacco smoke contains carbon monoxide which will lower the oxygen saturation of hemoglobin in healthy gingiva resulting in a significant reduction of oxygen tension within the periodontal pockets in smokers [171]. This decrease in oxygen tension may favor the growth of anaerobic bacteria, even in the shallower pockets. Periodontitis is caused by over-expression of pro-inflammatory cytokines and inflammatory mediators at the periodontium. In smoking-associated periodontitis, bone loss occurs because of various osteolytic mediators such as IL-1β, IL-8, RANKL, MMP-2, MMP-9, and tPA.

1.9.a. Smoking and the host immune response to oral microorganisms:

The susceptibility of epithelial cells, the mechanical protective barriers of the tissue, to become colonized by periodontal pathogens is modulated by smoking [172]. In an *in vitro* study, it has been shown that cigarette smoke could impair epithelial innate immune responses to microbial products, allowing overgrowth and invasion [173]. Nicotine can

suppress osteoblast proliferation while stimulating alkaline phosphatase activity, enhancing the bone loss. The ability of tobacco products to decrease the proliferative capacity of T and B lymphocytes might contribute to this diminished production of protective antibodies [174]. Nicotine induces pathological changes in human periodontal tissue, resulting in altered morphology and structure of host cells, decreased bone volume, and tooth loss [163, 175-177]. Cigarette smoke also reduces the host response to periodontopathic bacteria, resulting in a more destructive periodontitis [178]. Numerous functions of oral or peripheral neutrophils are negatively affected by smoking or nicotine exposure, including phagocytosis, superoxide, and hydrogen peroxide generation, integrin expression, and protease inhibitor production [179]. Because of down-regulation of anti-inflammatory factors along with an upregulation of pro-inflammatory cytokines, there might be increased infection risk by periodontal pathogens in smokers. Smokers are susceptible to colonization by *P. gingivalis*, a causative agent of periodontitis [180].

1.9.b. Smoking and oral microflora:

There are conflicting reports on the effects of smoking on oral microflora with some studies reporting no differences between smokers and non-smokers in the detection of periodontal pathogens [181, 182]. Some reported that smoking has little effect on the subgingival microflora [183-187].

At the same time, studies have suggested that smokers harbor a greater number of periodontal pathogens than non-smokers [188-191]. Recent studies, using real-time PCR, have demonstrated a positive relationship between the degree of smoking and amount of bacteria and probing depth [192, 193]. In these investigations, the subgingival microbiome of smokers was examined by traditional methods such as culturing, and targeted DNA-based assays such as polymerase chain reaction (PCR), real-time PCR, and DNA–DNA hybridization.

Owing differences in the experimental design and interpretation of the results, there might be variations in the results. Microbiological studies showed that smokers had a higher prevalence of bacterial species related to periodontal disease compared to nonsmokers, including *P. gingivalis*, *Aggregatibacter actinomycetencomitans, Bacteroides forsythus, Prevotella intermedia,* and *Fusobacterium nucleatum.* Data from the large Erie County study showed that the proportions of subjects positive for *Aggregatibacter actinomycetamcomitans* [194], *P. gingivalis*, and *Tannerella forsythia [195]* were higher among smokers. The oral biofilm in smokers is distinct from that of nonsmokers by less diversity [62].The flora of smokers contains fewer aerobic and anaerobic organisms with interfering activity against bacterial pathogens, and harbors more potential pathogens. Smoking cessation can lead to a decrease in the prevalence or proportion of many periodontal pathogens, including *Porphyromonas endodontalis, Dialister pneumosintes, Parvimonas micra, F. alocis* and *T. denticola* [196]. Increased prevalence of the keystone pathogen *P. gingivalis*, has repeatedly been shown and infection is more persistent in smokers compared with non-smokers [197]. The adoption of 16S ribosomal RNA gene (rDNA) amplification by PCR, followed by cloning and sequencing, has allowed more comprehensive broad-range investigation of oral bacterial communities. This method allowed for the identification of many un-culturable unknown species in subgingival biofilm [198, 199]. These studies identified that the microbial profile of smokers with periodontitis is distinct from that of non-smokers and predominated by periodontal pathogens [158, 198, 200].

1.9.c. Cigarette smoke and *P. gingivalis*:

It is assumed that the oral cavities of smokers, including the oral tissues and their microbiota, are exposed to high concentrations of nicotine and cotinine [201]. Nicotine and cotinine concentrations are much higher in saliva and gingival crevicular fluid than in

plasma [202]. Nicotine is the abundant alkaloid and cotinine is the primary metabolite of nicotine. *In vitro* and *in vivo* studies have demonstrated that nicotine can affect various functions of human periodontal ligament fibroblasts, upregulate the lipopolysaccharide-mediated secretion of prostaglandin E_2 by monocytes, stimulate osteoclast resorption, augment cytokine levels in nicotine-treated mice and have other deleterious effects on the periodontal tissues [203-208]. Sayers *et al.* reported a synergic interaction between *P. gingivalis* toxins and nicotine or cotinine. The colonization of epithelial cells by *P. gingivalis* may also be altered in the presence of nicotine or cotinine [172, 209]. Nicotine and cotinine, have been shown to increase the lethality of cell-free extracellular toxins and cell lysates from *P. gingivalis* in a chick embryo model [210, 211]. The combination of benzopyrene, a tobacco smoke aryl hydrocarbon, and *P. gingivalis* lipopolysaccharide (LPS) significantly increase the inhibition of osteogenesis in a rat bone marrow cell model compared to either agonist alone [212]. In an *in vitro* model, periodontal ligament (PDL) cells treated with both nicotine and LPS increased the expression of MMP-1, MMP-2, MMP-3, PGE_2, cyclooxygenase-2 (COX-2) and tissue-type plasminogen activators in osteoblasts [213, 214]. It also stimulates the formation of osteoclast-like cells by increasing macrophage colony-stimulating factor and PGE_2 production in osteoblasts [215]. Nicotine and LPS synergistically induced the production of nitric oxide and PGE_2, and increased the expression of inducible nitric oxide synthase (iNOS) and COX-2 via heme oxygenase-1 in PDLCs [216]. From these studies, it is evident that the pro-inflammatory potential of *P. gingivalis* is reduced in the presence of nicotine. This synergy, between nicotine and *P. gingivalis* LPS, results in deregulation of several host immune responses which work to combat the invading bacteria, thus promoting the growth of other pathogenic bacteria.

Other studies explain the increased infection in smokers due to modification of host immunity, but few studies explain the influence of tobacco smoke on the agent factors (oral bacteria). Host and environmental stimuli may confer a selective growth advantage to pathogenic organisms. Bacterial adaptation to these environmental conditions might be the cause for the persistent bacterial infection in smokers [69].

Virulence of periodontal pathogens may vary as a result of differed gene expression of virulence factors specific to particular environmental conditions [69, 217]. In a study done by Bagaitkar *et al.*, it was shown that CSE (cigarette smoke extract) significantly influences the expression of 6.8% of the *P. gingivalis* genome, as determined by microarray analysis. This included genes regulating capsule and fimbriae, oxidative stress and, DNA repair. Regulation in response to CSE of *fimA* and capsule was further confirmed by an *in vitro* study, where there was a significant increase in homotypic biofilm formation in the presence of CSE [218].

P. gingivalis adapts to the environmental stress induced by CSE by altering the expression of several genes and outer membrane proteins [180]. To withstand these adverse effects, there might be upregulation of certain genes for the repair of damaged organelles. Despite information regarding various virulence factors of *P. gingivalis* and their host interactions at the phenotypic level, the genes essential for the survival of *P. gingngivalis* in smokers have not been identified.

1.10. Periodontal diseases and GDM:

Pregnancy is associated with the development of new periodontal disease and progression of periodontitis [219, 220]. The prevalence of gingivitis in pregnant women has reportedly ranged from 30% to 100% [220]. Globally, 40% to 60% of females experience gingivitis during their pregnancy [221]. The severity of pregnancy-related

gingivitis usually increases in the second trimester. Periodontal diseases are associated with pre-term labor and congenital disabilities [222]. GDM also has been associated with poor pregnancy outcomes including preeclampsia, cesarean delivery, premature rupture of membranes, and preterm delivery [223-226]. GDM is defined as any degree of glucose intolerance with an onset first recognized during pregnancy [227]. As per a Center for Disease Control and Prevention (CDC) report, the prevalence of GDM in the United States may be as high as 9.2%. The International Diabetes Federation (IDF), Diabetes Atlas 2015, estimates that one in seven births is affected by GDM. Women with gestational diabetes had a greater risk of developing more severe periodontal disease during pregnancy than those without [228, 229]. It has been shown that poor glycemic control results in increased subgingival infection with *P. gingivalis* and *T. denticola* [230]. Studies have indicated that treating periodontal disease in pregnancy can reduce the likelihood of pre-term birth. Others have found no such reduction for births at less than 37 weeks of gestation, but have suggested evidence of a benefit for births before 32 weeks [231-234].

Inflammatory mediators that occur in periodontal disease play a significant role in the initiation of labor [235]. Increased levels of C-reactive protein, TNF-α, and IL-6 among women with gestational diabetes mellitus suggest a role of infection and inflammation in its etiology [236-238]. In a study by Dasanayake *et al.,* women with gestational diabetes mellitus had levels of C-reactive protein significantly higher than those without. During pregnancy, accumulation of hormones in gingival tissues affects gingival vasculature, and the local immune system reaction to dental plaque [239].The increased level of progesterone during pregnancy may provide a favorable environment for certain gingivitis-causing bacteria. These increased levels of progesterone may also make gum tissue more sensitive to plaque, exaggerating the host response. Mittas *et al*. found that plaque accumulation during GDM might be the main reason for gingival inflammation.

Periodontal diseases may increase the systemic inflammatory challenge, due to hematogenous spread of bacteria and their products as a result of ulceration of epithelial cells in periodontal pockets [240, 241]. *P. gingivalis* and *T. denticola* were found at a significantly higher level among the pregnant women with periodontitis [221]. Recent metagenomic analysis studies revealed that placental microbiome profiles were similar to the oral microbiome [242]. *P. gingivalis* has been found in the amniotic fluid in addition to the subgingival plaque and [222]. *P. gingivalis* was detected in the placentas of pregnant women with periodontitis who had preterm deliveries [241]. There was exacerbated gingival inflammation in an animal model when pregnant mice were challenged with *P. gingivalis* [243]. In an animal model, *P. gingivalis*-derived LPS infected pregnant mice exhibited reduced fetal weight and increased fetal resorption [244]. Another periodontal pathogen, *T. denticola* has also been associated with pregnancy complications and poor pregnancy outcome [221, 222, 243, 245]. In a mouse model, it has been shown that *T. denticola* has an ability to disseminate to distant sites from the site of infection [246]. In a mouse subcutaneous model, it has been shown that the newly emerging periodontal pathogen, *F. alocis* has the ability to colonize and spread to distant sites [54].

There are many studies showing the relationship between periodontitis, GDM and poor pregnancy outcomes; periodontal diseases, GDM and oral pathogens; and the interrelationship between periodontitis and GDM. There are few studies that addressed: the relationship between pregnancy-associated gingivitis and oral pathogens; and GDM and oral pathogens. Numerous studies show that gingivitis in pregnant women can occur due to physiological condition rather than the accumulation of plaque. In chapter 2 we studied whether there exists any relationship between pregnancy gingivitis; GDM and the established periodontal pathogens, *P. gingivalis*, *T. denticola* and *F. alocis*.

CHAPTER 2

INCREASED INFECTION WITH KEY PERIODONTAL PATHOGENS DURING GESTATIONAL DIABETES MELLITUS

2.1. Introduction

Increased gingival inflammation is well documented during pregnancy [247-249], and periodontal diseases increase the risk for maternal (hypertension and preeclampsia) and fetal complications (low birth weight and pre-term birth) [250-256]. For example, active periodontal inflammation, as determined by bleeding on probing, has been reported to correlate with reductions in fetal femur length, birth weight and birth length [253]. Indeed, it has even been suggested that periodontal disease severity may predict adverse pregnancy outcome [257].

Gestational diabetes mellitus (GDM) occurs in 2 to more than 10% of pregnancies [258] and also increases the risk for pregnancy complications, such as placental abnormalities, pre-eclampsia, emergency cesarean delivery, and stillbirth, as well as future development of type 2 diabetes mellitus [259-263].

Diabetes and periodontal inflammation correlate, with overt diabetes associated with an increased risk of more severe periodontitis while periodontitis has been associated with worsened glycemic control in subjects with diabetes [264-267]. There are only a few studies that address the interrelationships of GDM and gingivitis during pregnancy, specifically, and exacerbation of gingival inflammation by gestational GDM [228, 268, 269].

Gingivitis is a bacterial-induced disease and specific dental plaque pathogens, including *P. gingivalis* and *Treponema denticola*, have been associated with poor

pregnancy outcomes [221, 222, 240, 243, 245]. Furthermore, poor glycemic control has been reported to result in increased subgingival infection with these same two pathogens [230]. Increased low-grade systemic inflammation has been suggested to be related to pregnancy complications, such as preeclampsia [54, 270], with elevated levels of the acute phase protein, C-reactive protein (CRP), suggested to be an effective early biomarker for GDM [271].

We hypothesized that both pregnancy-associated gingivitis and gestational diabetes mellitus would be associated with increased oral infection with key oral pathogens and we set out to monitor oral infection with *P. gingivalis*, *T. denticola,* and the recently emergent oral pathogen, *Filifactor alocis* [29], as well as the systemic inflammatory burden, in 117 pregnant women with and without gingivitis and with and without gestational diabetes.

2.2. Materials and Methods

Materials

Chelex 100 was purchased from Bio-Rad Laboratories (Hercules, CA, USA), PCR primers came from Bio-Synthesis Inc. (Lewisville, TX, USA), while PCR SuperMix and UltraPure distilled water were from (Invitrogen, Carlsbad, CA, USA). Gifu anaerobic medium (GAM) was purchased from Nissui Pharmaceutical (Tokyo, Japan) while brain heart infusion (BHI) medium came from Becton Dickenson (Sparks, MD, USA). Fetal bovine serum was obtained from Atlanta Biologicals (Lawrenceville, GA, USA). All other media components came from Sigma-Aldrich (St. Louis, MO, USA). High sensitivity cotinine immunoassays were obtained from Salimetrics (State College, PA, USA). Finally, CRP EIA kits were ordered from Cayman Chemical Company (Ann Arbor, MI, USA).

Study population

All individuals were recruited between September 2012 and March 2013 at the Endocrinology and Metabolism outpatient clinic, Aydın State Hospital, Aydın, Turkey. The study was conducted in full accordance with ethical principles, including the World Medical Association's Declaration of Helsinki, as revised in 2008. The protocol was approved by the Ethics Committee of Medical Faculty of Ege University (protocol number 13-2/9). The study protocol was explained and written informed consent was received from each individual before enrollment in the study. Medical and dental histories were obtained from each individual, and saliva and serum samples were obtained before the clinical periodontal examination. The inclusion criteria were being in the third trimester, and having no other diagnosed systemic diseases requiring usage of medications such as antibiotics, non-steroidal anti-inflammatory drugs, corticosteroids or drugs that cause gingival overgrowth. Smoking status was determined according to the self-reports of the pregnant women and then verified biochemically by salivary cotinine analysis. The exclusion criterion was a body mass index (BMI) value ≥30 (kg/m^2).

Gestational diabetes mellitus was diagnosed according to the current American Diabetes Association criteria [272], i.e. a 75 g oral glucose tolerance test was administered, with fasting plasma glucose measurement at 1 and 2 h, at 24–28 weeks of gestation in women not previously diagnosed with overt diabetes. GDM was diagnosed if the following threshold values were met: fasting, ≥92 mg/dl (5.1 mmol/l); 1 h, ≥180 mg/dl (10.0 mmol/l); and 2 h, ≥153 mg/dl (8.5 mmol/l).

Gingivitis was diagnosed as previously described [273-275], i.e., when bleeding on probing (BOP) was present at >50% of all sites and probing depth (PD) was <3 mm at ≥90% of the measured sites, no more than one site with a PD >4 mm and clinical attachment level (CAL) ≤1 mm. All participants had ≥20 teeth present. Periodontally

healthy women were required to present with <30% BOP, and no clinical sign of alveolar bone loss. Plaque index (PI) was recorded as present or absent following visual examination [276].

Saliva and serum sampling

Whole, unstimulated saliva samples were obtained, before any clinical measurement or periodontal intervention, by expectoration into polypropylene tubes in the morning, following an overnight fast, during which participants were requested not to drink (except water) or chew gum, essentially as previously reported [277]. The saliva samples were clarified by centrifugation (800 *g*) for 10 min at room temperature, and 500 *μ*l amounts were placed in polypropylene tubes, and immediately lyophilized Venous blood (5 ml) was taken from the antecubital vein by standard venipuncture. Serum was separated by centrifugation at 1500 *g* for 10 min and immediately frozen at −40°C then lyophilized. Samples were shipped to the University of Louisville for biochemical and microbiological analysis. Ethical approval to analyze biomedical samples originating in Turkey was obtained from the Institutional Research Board of the University of Louisville (protocol number 13.0670).

Determination of systemic CRP concentrations

Circulating CRP was measured in serum using EIA kits, according to the manufacturer's instructions (Cayman Chemical Co.), using an Emax Precision Microplate Reader (Molecular Devices, Sunnyvale, CA, USA). The reported sensitivity of the assay is 50 pg/ml.

Determination of salivary cotinine concentrations

Salivary cotinine was measured using a high sensitivity cotinine immunoassay (Salimetrics) and an Emax Precision Microplate Reader, as previously reported [277]. The reported sensitivity of the assay is 0.15 ng/ml.

Growth of periodontal pathogens

P. gingivalis ATCC 33277; *F. alocis* ATCC 38596; and *T. denticola* ATCC 35405 were grown to mid-to-late log phase at 37°C (80% N_2, 10% H_2, 10% CO_2) in GAM; BHI supplemented with FBS (5%), L-cysteine (0.1%) and arginine (20%); and new oral spirochete broth (with veal heart infusion replaced with BHI and KH_2PO_4 omitted) [278], respectively, in an anaerobic chamber (Coy Laboratories, Grass Lake, MI, USA).

Detection of pathogens

The presence or absence of bacterial DNA was determined by four PCR amplifications per Chelex 100 (25% mass/volume)-treated saliva sample, using primers documented in Table 1. Cycling parameters were as previously reported [277, 279, 280]. DNA extracted from cultured periodontopathogens and water served as positive and negative controls, respectively. *P. gingivalis*, *T. denticola*, *F. alocis* and universal 16S rRNA amplicons were visualized using the Lonza Flashgel system (Rockland, ME, USA).

Primers	Forward Primer	Reverse Primer
Universal*	AGA GTT TGA TCC TGG CTC AG	ACG GCT ACC TTG TTA CGA CTT
P. gingivalis*	AGG CAG CTT GCC ATA CTG CG	ACT GTT AGC AAC TAC CGA TGT
F. alocis*	CAG GTG GTT TAA CAA GTT AGT GG	CTA AGT TGT CCT TAG CTG TCT CG
T. denticola*	TAA TAC CGA ATG TGC TCA TTT ACA T	TCA AAG AAG CAT TCC CTC TTC TTC TTA
Primer sequences and amplification conditions were first published by *[1] and **[3]		

Table 1: Primers used to detect bacterial DNA in saliva

Data analyses

The sample size was based on precedent literature examining the relationships between diabetes, pregnancy, CRP concentrations and infection with oral pathogens [229, 230, 240, 271, 281]. Statistical significance was determined using Instat v3.06 (GraphPad, San Diego, CA, USA). Parametric or nonparametric ANOVA with Tukey or Dunn post-testing, respectively, and Fisher's Exact Test were employed, as appropriate. Correlations between measurements of interest were determined by Spearman rank analysis. Significance was set at $p \leq 0.05$.

2.3. Results

The characteristics of all recruited women are shown in Table 2. The periodontally healthy group with GDM was older than the periodontally healthy group without GDM by an average of 3.4 years. As expected, all clinical parameters were higher in those with gingivitis compared to the periodontally healthy women while those with GDM had a higher BMI than those who did not. By chance, the groups were matched for smoking (smoking

yes *versus* no). Furthermore, there was no correlation between cotinine concentration and BoP (p = 0.636), perhaps due to the small number of smokers and low cotinine concentrations.

	Age	Smoking status	Cotinine (ng/ml)	Plaque (%)	Bleeding (%)	Pocket depth (mm)	BMI (kg/m²)
No Gingivitis No GDM (n = 27)	28.7 (4.5)	3/27 (11.1%)	5.5 (14.6)	20.4 (1.9)	20.4 (1.9)	1.0 (0.2)	21.7 (2.3)
Gingivitis No GDM (n = 31)	28.2 (4.5)	3/31 (9.6%)	8.3 (25.7)	72.3 (13.3)***	69.0 (14.0)***	2.4(0.5)***	21.1 (3.5)
No Gingivitis GDM (n = 21)	33.1 (5.0)#	4/21 (19.0%)	31.7 (81.7)	20.0 (0)	20.0 (0)	1.0 (0.0)	27.4 (4.1)###
Gingivitis GDM (n = 38)	31.0 (4.9)	5/38 (13.2%)	22.3 (66.9)	72.6 (17.5)***	70.3 (18.4)***	2.6 (0.6)***	28.2 (5.5)***

#$p \leq 0.05$; ***/###$p \leq 0.001$.

*Gingivitis *versus* no gingivitis; #GDM *versus* No GDM.

Table 2: Characteristics of the study population

Women with gingivitis during pregnancy are more likely to harbor periodontopathogens

As expected, because gingivitis is a bacteria-induced disease, pregnant subjects with gingivitis were more likely to harbor periodontopathogens than those who were periodontally healthy, as shown in Table 3.

	P. gingivalis	*F. alocis*	*T. denticola*	>1	All 3
No Gingivitis No GDM ($n = 27$)	6 (22.2%)	13 (48.1%)	20 (74.0%)	13 (48.1%)	4 (14.8%)
Gingivitis No GDM (n = 31)	13 (41.9%)**	21 (67.7%)***	19 (61.3%)	19 (61.3%)**	8 (25.8%)
No Gingivitis GDM (n = 21)	7 (33.3%)	16 (76.2%)$^{\#\#}$	13 (61.9%)	14 (66.6%)$^{\#\#}$	3 (14.3%)
Gingivitis GDM (n = 38)	20 (52.6%)**/$^{\#}$	29 (76.3%)	33 (86.8%)***/$^{\#\#\#}$	28 (73.6%)	16 (42.1%)$^{\#}$

$^{\#}p \leq 0.05$; **/$^{\#\#}p \leq 0.01$; ***/$^{\#\#\#}p \leq 0.001$.

*Gingivitis *versus* no gingivitis.

†GDM *versus* No GDM.

Table 3: Infection with periodontal pathogens.

Women with gestational diabetes mellitus are more likely to harbor periodontopathogens

Gestational diabetes mellitus itself was associated with increased infection with *P. gingivalis*, *F. alocis*, *T. denticola*, or a multiplicity of infection of individuals both with and without gingivitis, as shown in Table 3.

Gingivitis dramatically elevates systemic CRP in pregnant women

The systemic inflammatory burden, as determined by CRP measurement, was higher in those with gingivitis only compared to those without gingivitis (with or without GDM,

both $p < 0.01$) but not those with gingivitis and GDM, as shown in Figure 1. The mean (SD) CRP concentrations for those without gingivitis or GDM, with gingivitis only, with GDM only and those with both gingivitis and GDM, was 2495 (3007); 8386 (10006); 1838 (1389); and 8698 (4938) ng/ml, respectively. There was no correlation between cotinine concentration and CRP ($p = 0.835$), again perhaps due to the small number of smokers and low cotinine concentrations. Similarly, there was no correlation between BMI and CRP ($p = 0.265$) for the total population, likely reflecting the importance of gingival inflammation.

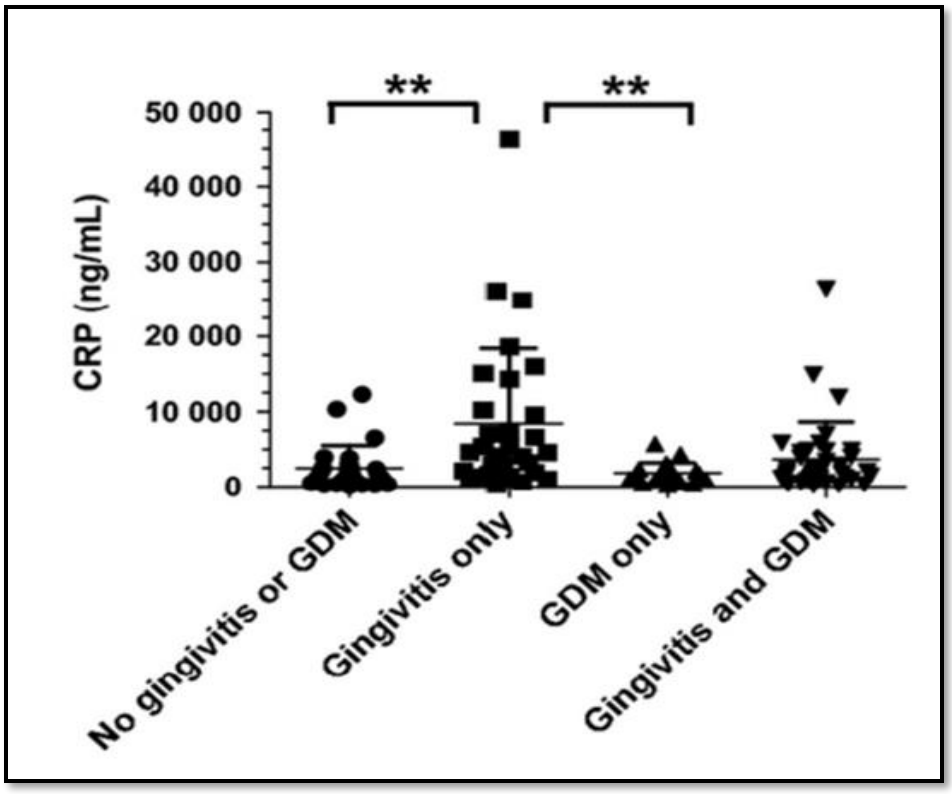

Figure 1: Systemic Inflammation (CRP) is elevated in pregnant women with gingivitis. The systemic CRP burden in women with and without gingivitis and with and without GDM are presented. The line and error bars represent mean (SD) values. **p≤0.001.

2.4. Discussion

A recent systematic review of the available epidemiological evidence determined that maternal periodontitis is independently associated with adverse pregnancy outcome, but that interpretation of the data is impacted by disease definition [282], a conclusion supported by a recent study [283]. Increased bacterial infection and systemic inflammation related to moderate and severe periodontitis are each risk factors for poor pregnancy outcome [248, 284, 285]. Specific microbial signatures of the subgingival biofilm were able to distinguish between microbiomes of periodontal health, gingivitis and periodontitis and these profiles may be helpful in establishing the risk of disease [286]. Herein, we specifically address the interactions of a different type of periodontal disease, gingivitis, and GDM.

Infection with periodontal bacteria has been reported to be an important indicator of poor pregnancy outcome, particularly pre-term and/or low birth weight infants [240, 287-290]. *P. gingivalis* is an anaerobic, Gram-negative bacterium and a causative agent of chronic periodontitis that is associated with several systemic sequelae, including pregnancy complications [222]. Even at low colonization levels, *P. gingivalis* employs a variety of strategies to control the commensal microbiota and direct disease progression and is thus regarded as a keystone species [19]. *T. denticola*, a highly motile, invasive, Gram-negative anaerobe, is also a causative agent of periodontitis that has been associated with pregnancy complications [245]. *F. alocis* is an anaerobic, Gram-positive bacterium, that has recently been identified by human microbiome projects as a pathogen associated with chronic periodontitis, aggressive periodontitis and endodontic lesions [56]. The potential relationship between *F. alocis* and maternal or fetal health, to the best of our knowledge, has not been examined.

From a mechanistic perspective, *P. gingivalis* has been the most extensively studied oral microbe. For example, injection of anti-*P. gingivalis* antibodies that are cross-reactive with cardiolipin into the tails of pregnant mice have been shown to increase fetal loss, relative to control antibody [291]. Administration of *P. gingivalis*-derived LPS to mice reduces fetal weight and increases fetal resorption [244]. Furthermore, *P. gingivalis* DNA has been found in chorionic tissues of high-risk pregnant women [292] and in the amniotic fluid of women bearing pre-term and/or low birth weight infants [240].

Saliva collection is more convenient, and thus likely to facilitate more ready recruitment than the isolation of subgingival dental plaque samples, while there are multiple reports that subgingival bacteria can be readily monitored in saliva and, furthermore, that bacteria detected in saliva correlate with periodontal disease status [293-295]. Therefore, saliva, rather than plaque, was employed herein. A high rate of infection with the emerging pathogen, *F. alocis* (48.1–76.3%) and the established but understudied pathogen, *T. denticola* (61.3–86.8%) was observed in all subject groupings. Despite this high background, gingivitis during pregnancy increased infection with periodontal pathogens in women with and without GDM. In those without GDM, gingivitis was associated with an increased frequency of detection of DNA from *P. gingivalis*, *F. alocis*, and combinations of the three targeted bacteria. In those with GDM, gingival inflammation resulted in increased infection with *P. gingivalis*, *T. denticola*, and combinations of the three targeted bacteria.

GDM itself increased the likelihood of infection with periodontopathogens in those with and without gingivitis during pregnancy. In those without gingivitis, GDM was associated with an increased frequency of detection of DNA from *F. alocis* and combinations of the three targeted bacteria. In those with gingivitis, GDM was associated with increased infection with *P. gingivalis*, *T. denticola*, and combinations of the three targeted bacteria.

Thus, gingivitis and GDM each increase the incidence of infection with periodontopathogens, an established risk factor for pregnancy complications.

Increased systemic inflammation has been suggested to be related to pregnancy complications, such as preeclampsia [54, 270], with elevated levels of the acute phase protein, C-reactive protein (CRP), reported to be an effective early biomarker for GDM [271]. All women were recruited during the third semester, the stage of pregnancy when CRP levels have been reported to be highest in those with GDM [237]. In women with periodontitis and pre-eclampsia, [296] it was found that CRP levels reflected periodontal disease severity. Here, we establish that CRP, employed as a biomarker of systemic inflammation, is dramatically elevated in women with gingivitis during pregnancy, irrespective of GDM diagnosis. CRP concentration is hypothesized to be related to fat deposition and BMI only in early pregnancy, with impaired glucose metabolism important only later in gestation [237, 297, 298]. Therefore, it is important to note that enrolled women with GDM had significantly higher mean BMI than those without GDM. In baboons, [299] have reported that immunoglobulin responses to oral bacteria (*Fusobacterium nucleatum*, *P. gingivalis*, *Aggregatibacter actinomycetemcomitans* and *Campylobacter rectus*) were associated with systemic inflammation during ligature-induced periodontitis in pregnancy [299].

It will be important to confirm these results in a larger population to ensure that the sample size (four groups of 21–38 subjects each) has not affected results such as CRP-BMI or smoking-CRP associations. In conclusion, and considering this limitation, these data suggest a bidirectional relationship between GDM and gingivitis, although, it has yet to be established if this concerted influence results in poor pregnancy outcome per se. Nevertheless, GDM and gingivitis are each associated with increased infection from pathogenic oral bacteria, while gingivitis increases systemic inflammation, irrespective of

GDM. The present study focuses on microbiological data and CRP level with regard to GDM and gingivitis. Further biochemical analysis of cytokine and collagenase levels are planned in the same study population.

Unfortunately, while intensive oral hygiene can be effective in decreasing gingivitis during pregnancy [300], women appear to receive less than their normal dental care when pregnant [301]. If confirmed, these results suggest that increased efforts to control gingivitis and glycemic control during pregnancy may help to reduce two important risk factors for poor pregnancy outcomes.

CHAPTER 3

COMPARISON OF INHERENTLY ESSENTIAL GENES OF *PORPHYROMONAS GINGIVALIS* IDENTIFIED IN TWO TRANSPOSON-SEQUENCING LIBRARIES

3.1. Introduction

Periodontitis, a microbially-driven, destructive disease of the tissues surrounding the teeth occurs in approximately 50% of the population [302]. Increasing evidence suggests that periodontitis is associated with elevated risk of vascular diseases, including coronary artery disease and stroke, diabetes mellitus, lung diseases such as chronic obstructive pulmonary disease and pneumonia, rheumatoid arthritis, preterm low birth weight delivery, and even some forms of cancer [303-307].

The anaerobic, Gram-negative bacterium *P. gingivalis* is an emerging systemic pathogen and a causative agent of chronic periodontitis. *P. gingivalis* has been proposed as the prototypic oral keystone pathogen [17], that is, a bacterium that promotes dysbiosis and inflammation-driven tissue destruction even at low levels of infection. *P. gingivalis* ATCC 33277 is one of the most commonly employed strains in periodontal research and is pathogenic in experimental animals following oral infection [137]. The *P. gingivalis* ATCC 33277 genome is comprised of 2155 genes, 2090 of which encode proteins, and there are no plasmids [32, 147, 148]. Recently, Klein *et al* [148] generated a Mariner transposon insertion mutant (TnSeq) library (which we denote Library 1) for this *P. gingivalis* strain and used it to identify 463 genes potentially required for *in vitro* viability. We have independently generated a TnSeq mutant library for *P. gingivalis* ATCC 33277 (Library 2), building on our previous work on *Vibrio* and *Pseudomonas* gene fitness [308-310].

There is no fully defined medium for *P. gingivalis*, and different complex media are likely to provide different nutritional environments. Therefore, we hypothesized that multiple

genes essential for planktonic growth in complex media would be common (i.e. potentially representing the core genome) and divergent (i.e. environment-specific) between Library 1 and Library 2.

3.2. Materials and Methods

Materials

P. gingivalis ATCC 33277 was cultured in Gifu anaerobe medium (GAM; Nissui Pharmaceutical, Tokyo, Japan) and on GAM blood agar. *Escherichia coli* DH5α and pSAM_Bt were cultured in Luria–Bertani broth and agar from BD Biosciences (San Jose, CA). Sheep blood was from Lampire Biological Laboratories (Pipersville, PA). Gentamicin, erythromycin, ampicillin, dichloromethane, sodium sulfate and analytical nicotine standards were from Sigma-Aldrich (St. Louis, MO). GeneReleaser® came from Bio Ventures, Inc. (Murfreesboro, TN). All primers were from Biosynthesis Inc. (Lewiston, TX). Lonza flash gels came from Lonza (Rockland, ME). Wizard SV and PCR clean-up kit were from Promega (Madison, WI). T4 DNA Ligase and buffer; *Mme*I; and TAE buffer came from New England Biolabs (Ipswich, MA), while HiFi Hotstart Readymix was from KAPA Biosystems (Wilmington, MA) and the polymerase chain reaction (PCR) SuperMix came from Invitrogen (Carlsbad, CA).

Generation and validation of a *P. gingivalis* ATCC 33277 transposon sequencing library

An ATCC 33277 mariner transposon insertion library was generated. *P. gingivalis* was inoculated into GAM without antibiotics; *E. coli* strain DH5α containing the pSAM_Bt plasmid was grown in Luria–Bertani broth with ampicillin; and a 5:1 ratio of log phase *P. gingivalis* to *E. coli* was employed for conjugation. pSAM_Bt was originally employed to generate the first TnSeq library in the porphyromonad-related

microbe, *Bacteroides thetaiotaomicron* [311]. Gentamicin (*E. coli* is sensitive; *P. gingivalis* is naturally resistant) and erythromycin (resistance is transposon-encoded) were used to select for insertion mutants grown on GAM/blood agar plates. Random insertion in the *P. gingivalis* genome was confirmed by nested semi-random PCR. One hundred randomly selected colonies were harvested to test for hot spots of insertion of the transposon by semi-nested PCR involving two rounds of PCR. DNA was harvested from selected colonies by GeneReleaser® following the manufacturer's protocol. Round one PCR was performed with Invitrogen Supermix using Round 1-pSAM and Round 1-RndomPG primers (Table 7) under the following conditions: 94°C for 3 min; 94°C for 30 s, 50°C for 40 s, 72°C for 3 min, 10 cycles; 94°C for 30 s, 62°C for 40 s, 72°C for 3 min, 25 cycles. Round one PCR products were used for round two PCR amplification using Round 2-PG and Round 2-pSAM primers (Table 7) under the following conditions: 94°C for 3 min; 94°C for 30 s, 57°C for 40 s, 72°C for 3 min, 30 cycles; 72°C for 5 min. Amplicons were run on a Lonza flash gel system to verify correct fragment size. 16S rRNA gene-specific primers (Table 7) were also used to confirm *P. gingivalis* specificity with the same cycle conditions as round 2 of the nested PCRs employed.

Identification of essential genes for planktonic growth in GAM

The mutant library was passaged twice after the culture reached mid-late log phase in 1 liter of GAM broth containing gentamicin (50 μg ml^{-1}) and erythromycin (5 μg ml^{-1}). Genomic DNA from the mutant library (100 μg) was harvested using the Wizard DNA isolation kit and digested with *Mme*I (1 h at 37°C; 20 min at 80°C). The DNA digest was run on a 1% agarose gel and the band corresponding to the transposon plus the flanking sequences (~1500) was extracted using a Wizard gel extraction kit, as per the manufacturer's instructions. A double-stranded DNA adapter was created using the primers LIB_AdaptT_4 and LIB_AdaptB_4 (Table 7). The primers were combined and

heated at 95°C for 5 min and then allowed to cool to room temperature. The dsDNA adapter was ligated to the gel purified DNA product using T4 DNA ligase (16 h at 16°C; 10 min at 65°C). Ligation products purified by a Wizard purification kit and amplified by PCR using HiFi Hotstart SuperMix with LIB_PCR_5 and LIB_PCR_3 primers (Table S1) under the following conditions: 98°C for 3 min; 98°C for 20 s; 60°C for 1 min; 72°C for 1 min, 18 cycles; 72°C for 4 min. The PCR product was purified using a Wizard purification kit and run on a Lonza flash gel system to verify the 125-base-pair band. Products were quantified using a NanoDrop ND-1000 spectrophotometer (Wilmington, DE) and sequenced at the University of Michigan DNA Core. The sequences were read by 50-base-pair single end reads. The prepared mutant library DNA was run on a single flow cell lane on the Illumina HiSeq2000 platform. The resulting reads were analyzed by CLC Genomics Workbench 7.1.1. Adapter sequences were removed along with the sequence corresponding to the end of the transposon. All sequences with reads with a quality score <0.05 or of <15 nucleotides were removed. The remaining reads were aligned to the annotated gene list of *P. gingivalis* strain ATCC 33277. To control for variations in gene length and depth of coverage, normalized genes with <0.5 reads per kilobase per million (RPKM) mapped reads were considered essential. The remaining genes were determined to be non-essential for survival in GAM broth.

Cross library comparison of essential genes for planktonic growth in complex media

The genes essential for survival in GAM broth were compared with the previously published list of essential genes for survival in supplemented brain–heart infusion broth [148]. The function and interrelationships of the 281 common inherently essential genes for planktonic growth of *P. gingivalis* in complex media were investigated *in silico* by KEGG (www.genome.jp/kegg) database analyses. The common essential genes were

mapped to the *P. gingivalis* ATCC 33277 genome using CGViewer (Stothard.afns.ualberta.ca/cgview_server/) [312] and compared with the Database of Essential Genes (DEG; tubic.tju.edu.cn/deg/, blastx search, <1e-8) [313].

3.3. Results

Random insertion in the *P. gingivalis* ATCC 33277 genome, which contains ~117,000 TA sites distributed among 2155 genes, was confirmed by nested semi-random PCR, as shown in Figure 2. Inherently essential genes common to Library 1 and Library 2 were mapped to the *P. gingivalis* ATCC 33277 genome using CGViewer, as presented in Figure 3. KEGG analysis (www.genome.jp/kegg) was employed to functionally classify the same *P. gingivalis* gene set, as noted in Table 4. The highest percentage of essential genes found within the functional groupings was for those whose products are involved in protein export, 89% of which were essential under these conditions. Similar functional groupings were obtained using the DAVID database (https://david.ncifcrf.gov). Functional relationships between the common essential genes were determined, using KEGG, as shown in Fig. 4a–e. Finally, the complete database of 281 common genes is provided in Table 5. These have been entered into the Database of Essential Genes [313].

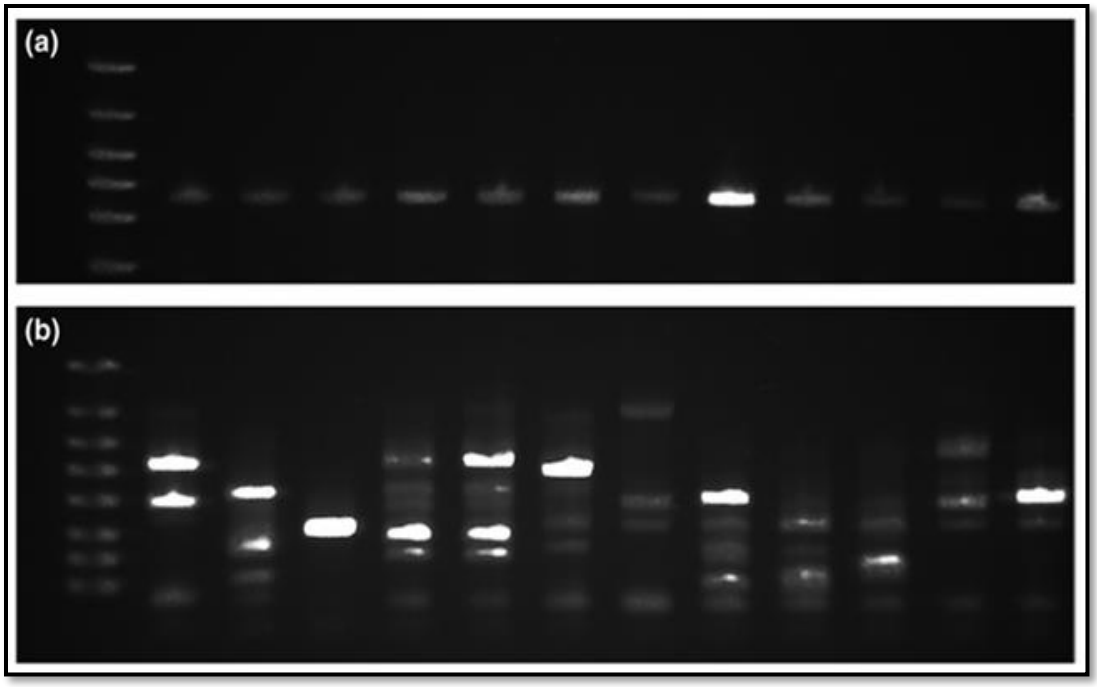

Figure 2: Validation of TnSeq library. (A) 16S rDNA confirmation of *P. gingivalis*; expected band size of 463 bp(B) random insertion determined by nested polymerase chain reaction (lanes from 100 randomly selected colonies). Approximately 80,000 insertion strains (individual mutants) were generated in the ATCC 33277 chromosome.

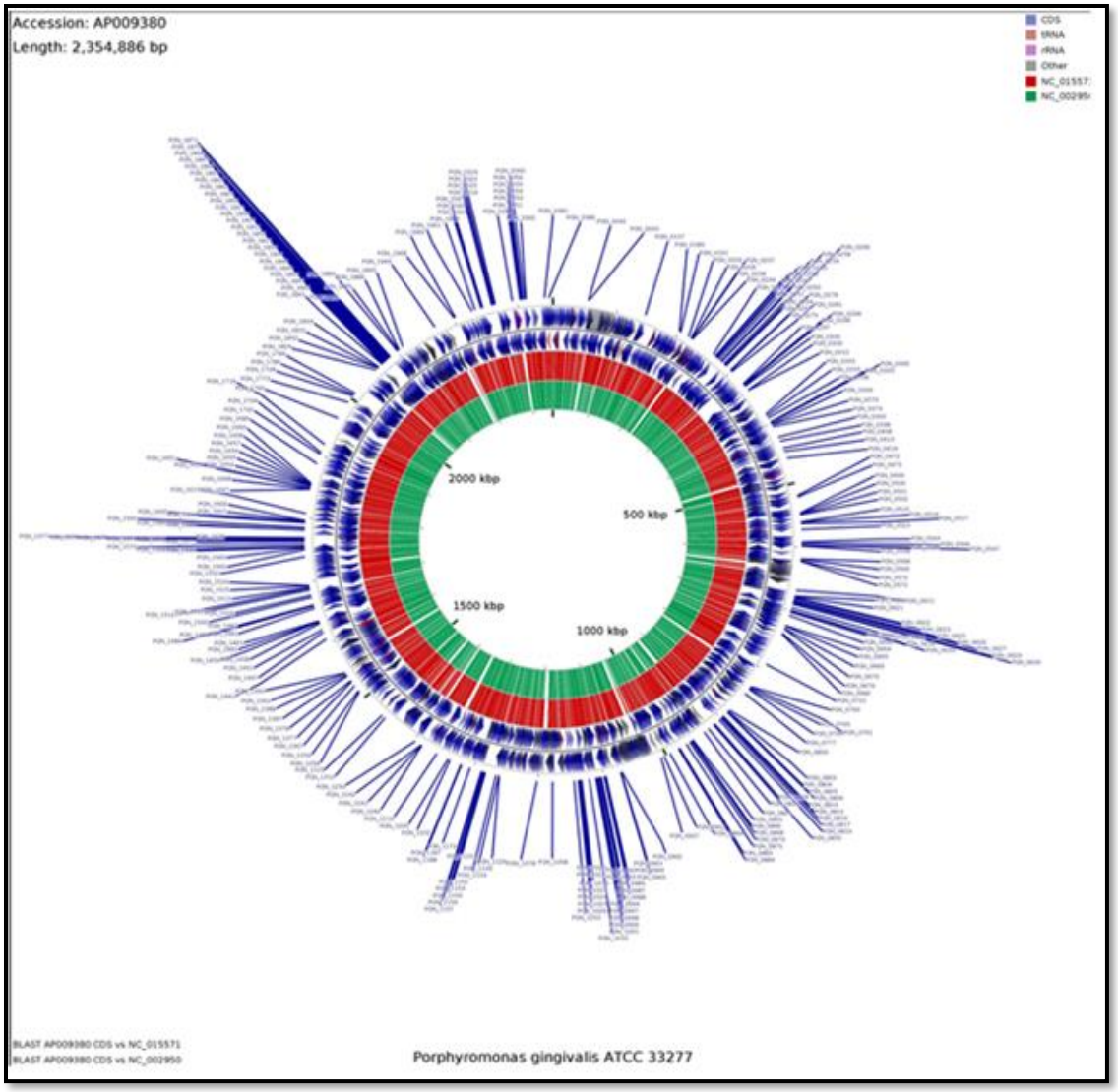

Figure 3: Map of putative common inherently essential genes for the planktonic growth of *P. gingivalis* in complex media. Common essential genes were mapped to the *P. gingivalis* ATCC 33277 genome using CG viewer (Grant& Stothard,2008). The blue arrows show the location of the 281-putative common essential genes within the *P. gingivalis* 33277 chromosome. The adjacent two rings depict gene orientation (outer and inner blue arrows represent the positive and negative strands, respectively). The next two rings represent the coding sequences for *P. gingivalis* TDC60 and W83 strains, respectively.

Cell envelope	32/108 (29.6%)
Fatty acid and phospholipid	3/15 (20.0%)
Protein fate	17/77 (22.1%)
Protein synthesis	60/110 (54.5%)
Purines, pyrimidines	13/45 (28.9%)
Regulatory functions	4/32 (12.5%)
Signal transduction	1/9 (11.1%)
Transcription	10/34 (29.4%)
Cell wall biogenesis	8/15 (53.3%)
Lipopolysaccharide biosynthesis	8/18 (44.4%)
Protein export	8/9 (89%)*
Riboflavin	2/5 (40.0%)
Ribosomal	32/54 (59.3%)
tRNA synthetase	15/24 (62.5%)

* The essential protein export genes, as determined by KEGG analysis, were *secY* (PGN_1848, PG1918, PGTDC60_0188); *secE* (PGN_1577, PGTDC60_1503); *secG* (PGN_0258, PGDTDC60_0422); *secD/F* (PGN_1702, PG1762, PGTDC60_1374); *yajC* (PGN_1485, PG0485, PGTDC60_1601); *SecA* (PGN_1458, PG0514, PGTDC60_1633); *SRP54/ffh* (PGN_1205, PG1115, PGTDC60_1100); *ftsY* (PGN_0264, PG0151, PGTDC60_0428). *yidC* (PGN_1446, PG0526, PGTDC60_1645) was found to be non-essential. The orthologues in *P. gingivalis* W83 and *P. gingivalis* TDC60, respectively, are also presented.

Table 4: Functional classification of inherently essential genes of *P. gingivalis* common to two transposon sequencing libraries based on the KEGG analysis.

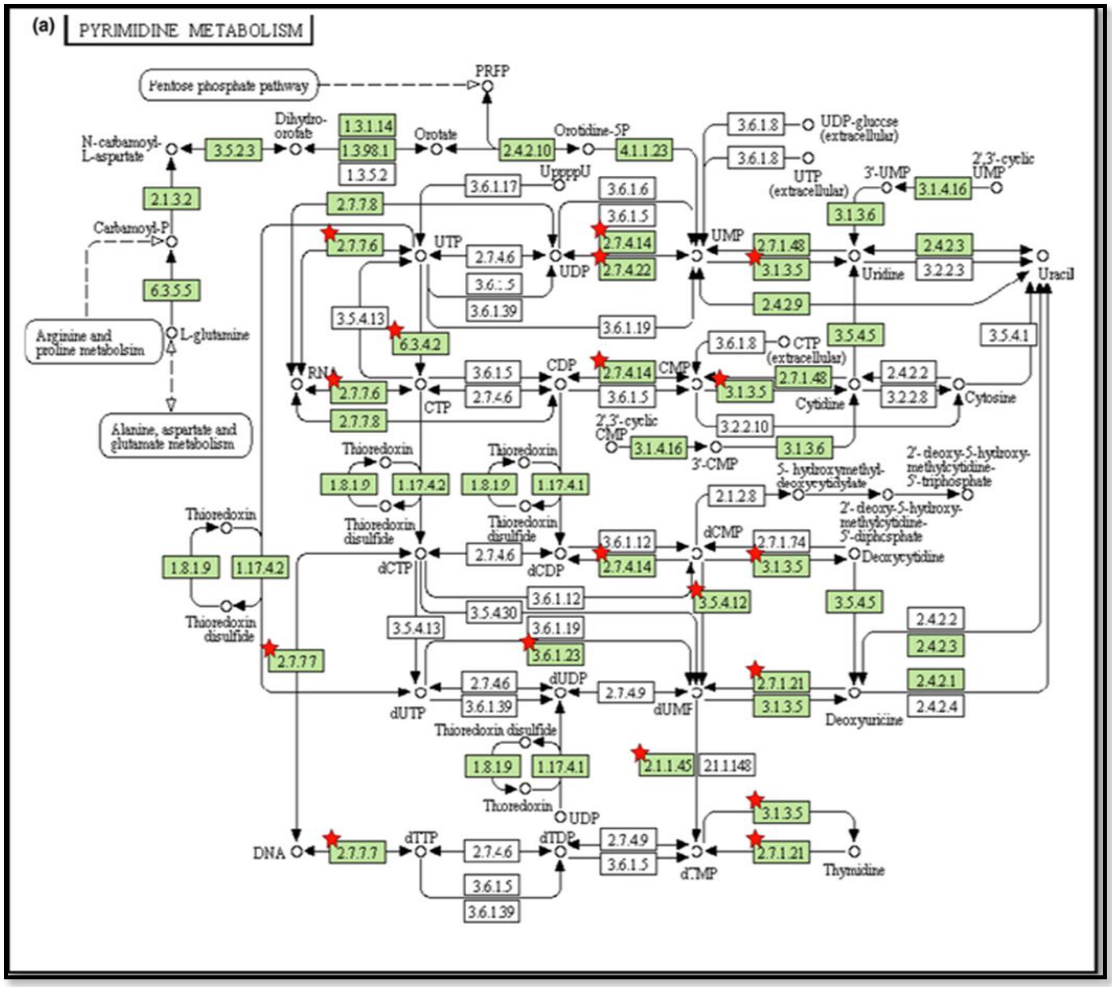

Figure 4a. Pyrimidine Metabolism (pathway enriched among inherently essential *P. gingivalis* genes). KEGG pathways (www.genome.jp/kegg) are presented with the proteins catalyzing each step shown by their enzyme commission number and substrates/products. Green shading indicates the presence of homologs in *P. gingivalis*. The red star denotes essential genes. The PGN assignments of the essential metabolic genes are provided in Table 9.

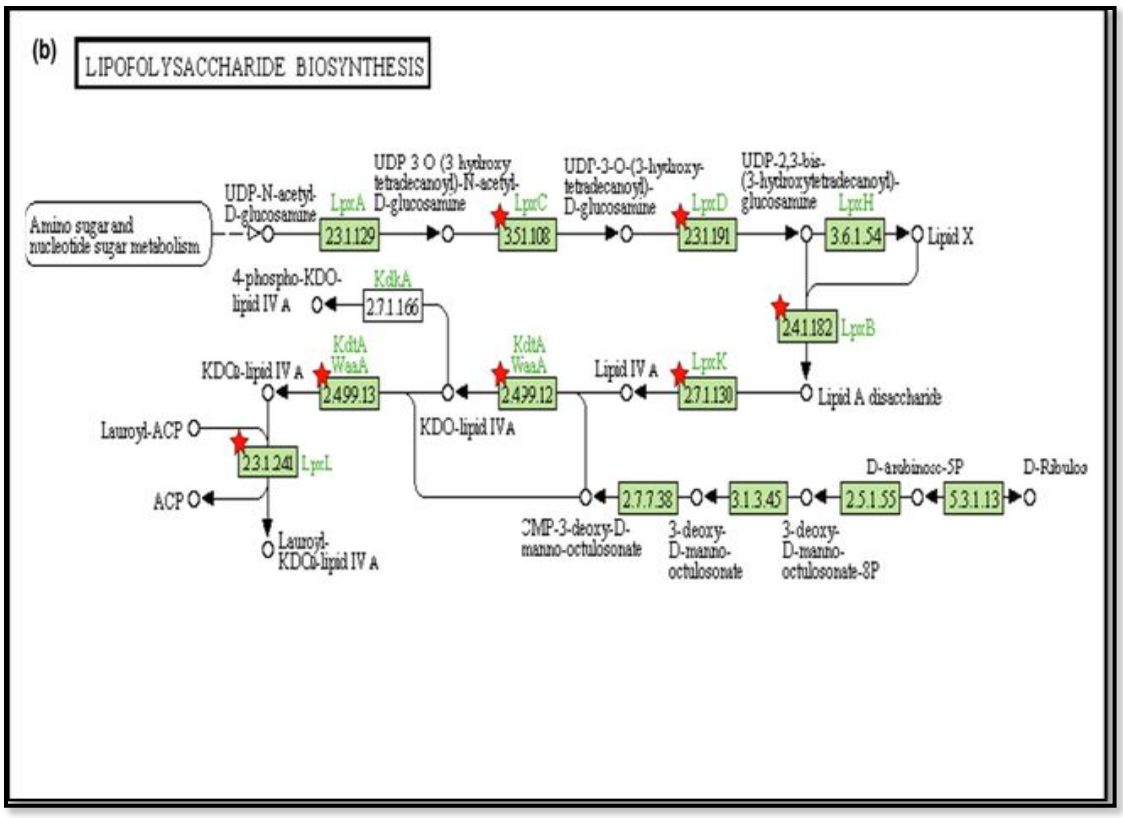

Figure 4b. Lipopolysaccharide Biosynthesis (pathway enriched among inherently essential *P. gingivalis* genes). KEGG pathways (www.genome.jp/kegg) are presented with the proteins catalyzing each step shown by their enzyme commission number and substrates/products. Green shading indicates the presence of homologs in *P. gingivalis*. The red star denotes essential genes. The PGN assignments of the essential metabolic genes are provided in Table 9.

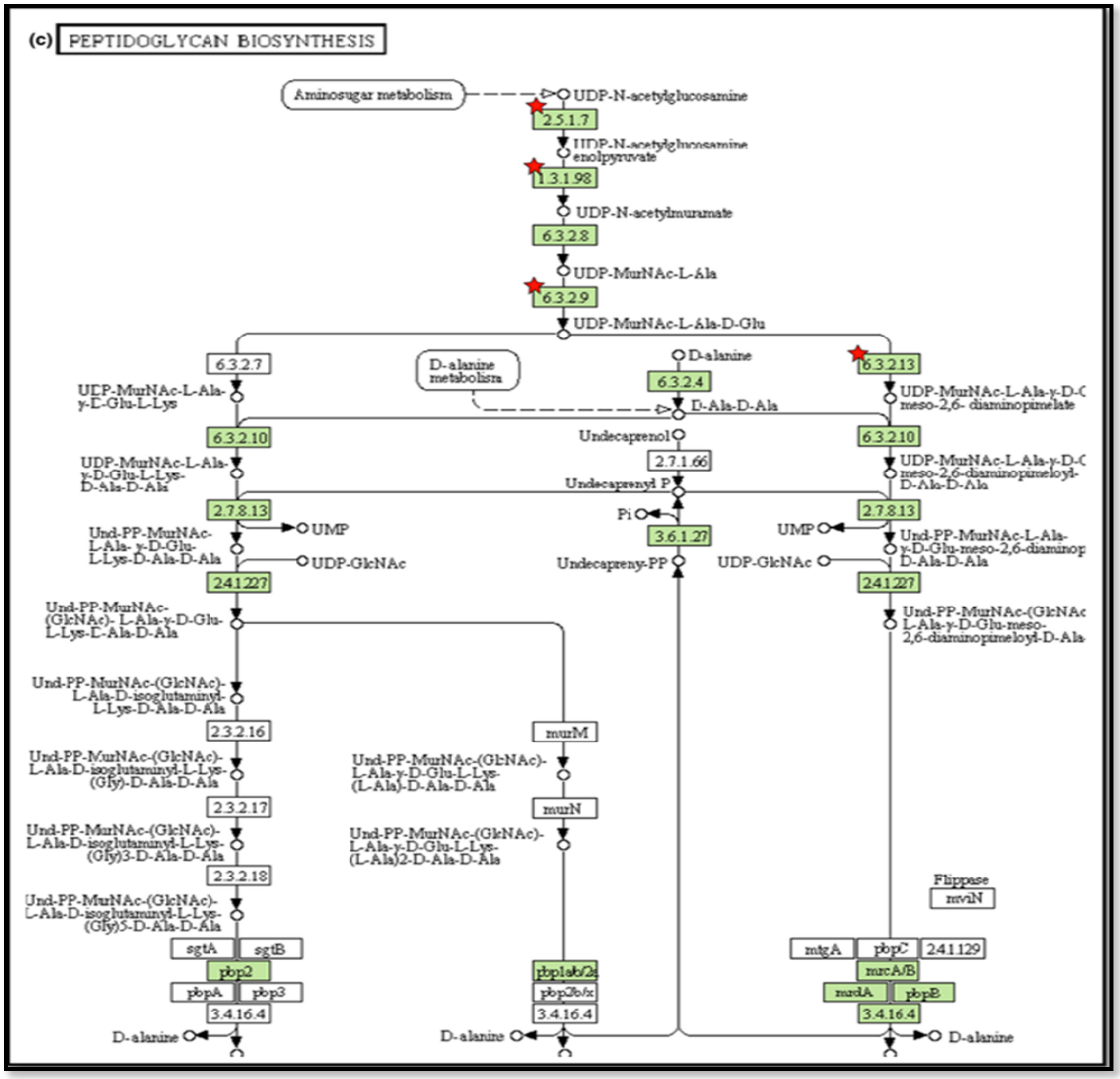

Figure 4c. Peptidoglycan Biosynthesis (pathway enriched among inherently essential *P. gingivalis* genes). KEGG pathways (www.genome.jp/kegg) are presented with the proteins catalyzing each step shown by their enzyme commission number and substrates/products. Green shading indicates the presence of homologs in *P. gingivalis*. The red star denotes essential genes. The PGN assignments of the essential metabolic genes are provided in Table 9.

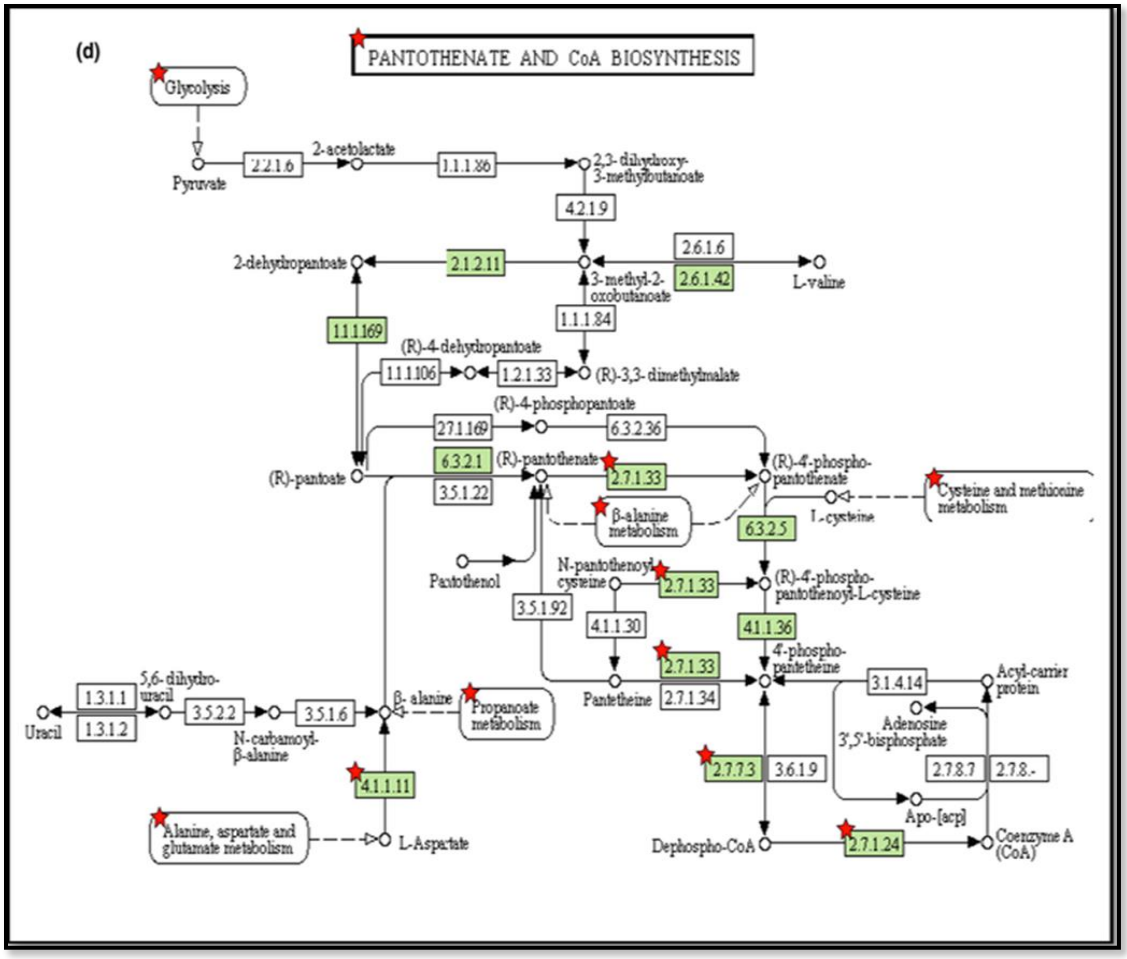

Figure 4d. Pantothenate and CoA Biosynthesis (pathway enriched among inherently essential *P. gingivalis* genes). KEGG pathways (www.genome.jp/kegg) are presented with the proteins catalyzing each step shown by their enzyme commission number and substrates/products. Green shading indicates the presence of homologs in *P. gingivalis*. The red star denotes essential genes. The PGN assignments of the essential metabolic genes are provided in Table 9.

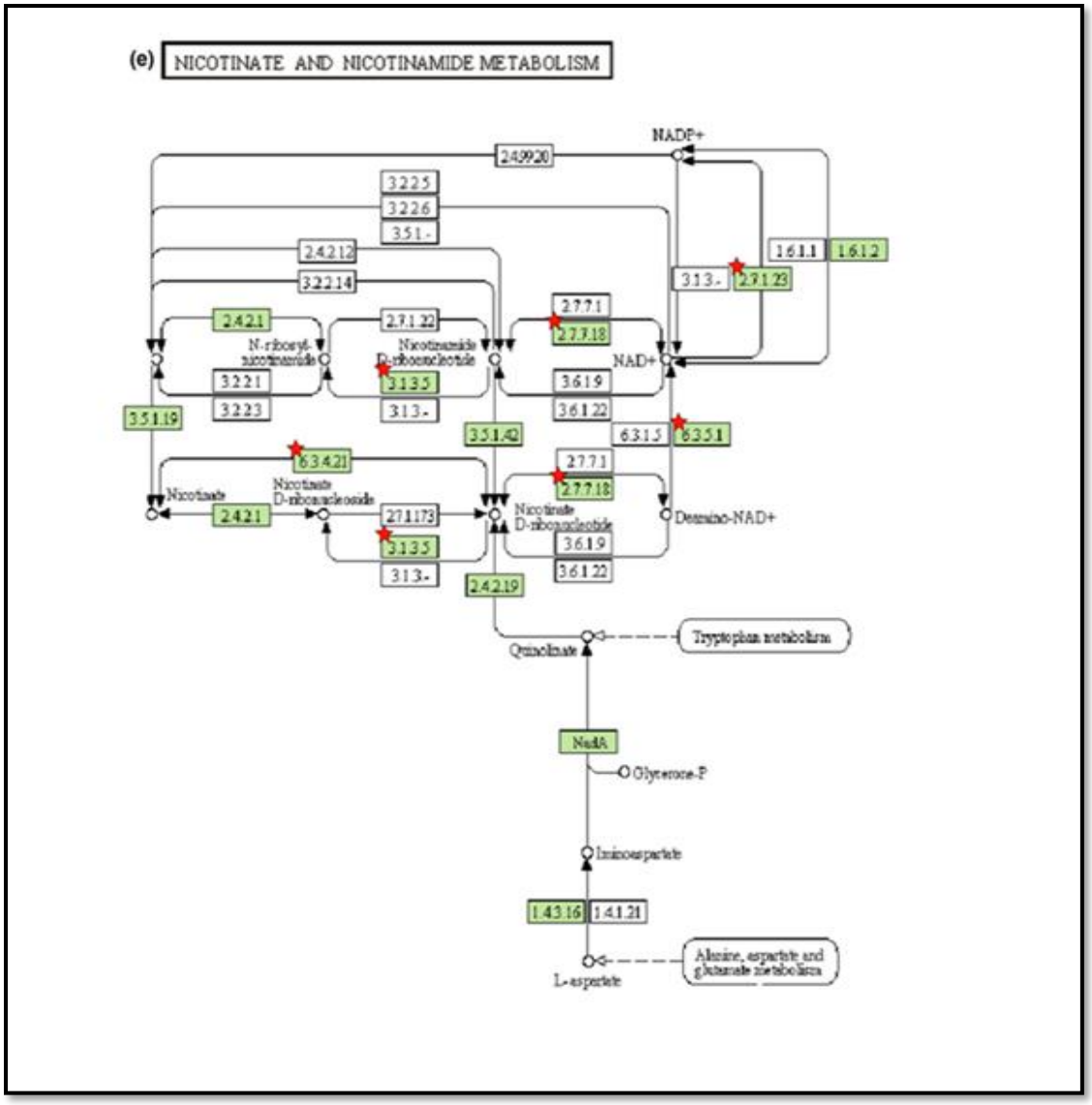

Figure 4e. Nicotinate and Nicotinamide Metabolism (pathway enriched among inherently essential *P. gingivalis* genes). KEGG pathways (www.genome.jp/kegg) are presented with the proteins catalyzing each step shown by their enzyme commission number and substrates/products. Green shading indicates the presence of homologs in *P. gingivalis*. The red star denotes essential genes. The PGN assignments of the essential metabolic genes are provided in Table 9.

PGN_0042	PGN_0043	PGN_0137	PGN_0189	PGN_0191	PGN_0202	PGN_0206	PGN_0207	PGN_0208
PGN_0209	PGN_0243	PGN_0247	PGN_0248	PGN_0250	PGN_0251	PGN_0254	PGN_0258	PGN_0260
PGN_0264	PGN_0266	PGN_0267	PGN_0278	PGN_0279	PGN_0281	PGN_0297	PGN_0298	PGN_0299
PGN_0300	PGN_0309	PGN_0310	PGN_0353	PGN_0355	PGN_0358	PGN_0365	PGN_0366	PGN_0369
PGN_0370	PGN_0374	PGN_0393	PGN_0398	PGN_0408	PGN_0413	PGN_0419	PGN_0472	PGN_0473
PGN_0499	PGN_0500	PGN_0501	PGN_0502	PGN_0515	PGN_0516	PGN_0517	PGN_0523	PGN_0543
PGN_0544	PGN_0546	PGN_0547	PGN_0548	PGN_0568	PGN_0569	PGN_0572	PGN_0573	PGN_0611
PGN_0620	PGN_0621	PGN_0622	PGN_0623	PGN_0625	PGN_0626	PGN_0627	PGN_0629	PGN_0630
PGN_0635	PGN_0638	PGN_0639	PGN_0642	PGN_0646	PGN_0664	PGN_0665	PGN_0669	PGN_0670
PGN_0679	PGN_0680	PGN_0723	PGN_0760	PGN_0761	PGN_0765	PGN_0766	PGN_0777	PGN_0800
PGN_0803	PGN_0804	PGN_0805	PGN_0806	PGN_0814	PGN_0815	PGN_0816	PGN_0817	PGN_0819
PGN_0828	PGN_0830	PGN_0833	PGN_0841	PGN_0865	PGN_0866	PGN_0868	PGN_0870	PGN_0875
PGN_0883	PGN_0884	PGN_0894	PGN_0902	PGN_0907	PGN_0962	PGN_0963	PGN_0964	PGN_0965
PGN_0985	PGN_0987	PGN_0992	PGN_0993	PGN_0994	PGN_0997	PGN_0998	PGN_0999	PGN_1001
PGN_1010	PGN_1019	PGN_1020	PGN_1022	PGN_1023	PGN_1024	PGN_1025	PGN_1026	PGN_1033
PGN_1058	PGN_1078	PGN_1129	PGN_1130	PGN_1134	PGN_1151	PGN_1152	PGN_1153	PGN_1154
PGN_1156	PGN_1157	PGN_1173	PGN_1187	PGN_1188	PGN_1202	PGN_1205	PGN_1219	PGN_1240
PGN_1241	PGN_1242	PGN_1250	PGN_1312	PGN_1315	PGN_1356	PGN_1359	PGN_1367	PGN_1377
PGN_1378	PGN_1387	PGN_1388	PGN_1391	PGN_1393	PGN_1441	PGN_1447	PGN_1451	PGN_1458
PGN_1459	PGN_1460	PGN_1461	PGN_1481	PGN_1485	PGN_1486	PGN_1487	PGN_1500	PGN_1504
PGN_1510	PGN_1512	PGN_1513	PGN_1515	PGN_1516	PGN_1552	PGN_1562	PGN_1565	PGN_1566
PGN_1568	PGN_1570	PGN_1571	PGN_1572	PGN_1573	PGN_1575	PGN_1576	PGN_1577	PGN_1578
PGN_1588	PGN_1589	PGN_1593	PGN_1599	PGN_1600	PGN_1601	PGN_1602	PGN_1615	PGN_1647
PGN_1648	PGN_1650	PGN_1651	PGN_1654	PGN_1655	PGN_1656	PGN_1657	PGN_1658	PGN_1660
PGN_1689	PGN_1702	PGN_1704	PGN_1716	PGN_1749	PGN_1773	PGN_1784	PGN_1786	PGN_1789
PGN_1829	PGN_1832	PGN_1833	PGN_1834	PGN_1841	PGN_1842	PGN_1843	PGN_1845	PGN_1846
PGN_1848	PGN_1849	PGN_1850	PGN_1851	PGN_1852	PGN_1853	PGN_1855	PGN_1856	PGN_1857
PGN_1859	PGN_1861	PGN_1862	PGN_1864	PGN_1865	PGN_1866	PGN_1867	PGN_1868	PGN_1870
PGN_1871	PGN_1872	PGN_1883	PGN_1885	PGN_1886	PGN_1895	PGN_1946	PGN_1968	PGN_1969
PGN_1991	PGN_1996	PGN_2005	PGN_2006	PGN_2007	PGN_2019	PGN_2020	PGN_2022	PGN_2024
PGN_2045	PGN_2051	PGN_2053	PGN_2054	PGN_2055	PGN_2056	PGN_2060	PGN_2062	PGN_2081
PGN_2086								

Table 5: Inherently essential genes of *P. gingivalis* identified in two transposon sequencing libraries (n= 281).

Media	Composition
Supplemented brain-heart infusion broth (BHIHKSbcStgC) Library 1 (Klein *et al.* 2012)	Brain-heart infusion (unspecified concentration), yeast extract (1 mg/ml), hemin (5 µg/ml), menadione (0.5 µg/ml), sodium bicarbonate (1 mg/ml), sodium thioglycolate (0.25 mg/ml), cysteine (0.5 mg/ml). Gentamicin (25-50 mg/ml) and erythromycin (2-10 mg/ml) also employed.
Gifu Anaerobe Media (GAM) Library 2 (herein)	Peptic digest of animal tissue (10 mg/ml), papaic digest of soyabean meal (3 mg/ml), proteose peptone (10 mg/ml), digested serum (13.5 mg/ml), yeast extract (5 mg/ml), beef extract (2.2 mg/ml), liver extract (1.2 mg/ml), dextrose (3 mg/ml), H_2KO_4P (2.5 mg/ml), NaCl (3 mg/ml), soluble starch (5 mg/ml), L-cysteine hydrochloride (0.3 mg/ml), and sodium thioglycolate (0.3 mg/ml). Gentamycin (50 µg/ml) and erythromycin (5 µg/ml) also employed.

Table 6: Composition of (A) BHIHKSbcStgC and (B) GAM complex media employed for the growth of *P. gingivalis* ATCC 33277.

ID	Sequence(5’ -> 3’)
Pg16S-F	AGG CAG CTT GCC ATA CTG CG
Pg16S-R	ACT GTT AGC AAC TAC CGA TGT
LIB_PCR_5	CAA GCA GAA GAC GGC ATA CGA AGA CCG GGG ACT TAT CAT CCA ACC TGT
LIB_PCR_3	AAT GAT ACG GCG ACC ACC GAA CAC TCT TTC CCT ACA CGA CGC TCT TCC GAT CT
Round 1-RndomPG-1	GGC CAC GCG TCG ACT AGT AC NNNNNNNNN CGATG
Round 1-RndomPG-2	GGC CAC GCG TCG ACT AGT AC NNNNNNNNN GGTAG
Round 1-RndomPG-3	GGC CAC GCG TCG ACT AGT AC NNNNNNNNN CGAAG
Round 1-RndomPG-4	GGC CAC GCG TCG ACT AGT AC NNNNNNNNN CAGCAG
Round 1-RndomPG-5	GGC CAC GCG TCG ACT AGT AC NNNNNNNND CAGCAG
Round1-pSAM	TCG AGG GCG CGC CAA GCA G
Round2-PG	GGC CAC GCG TCG ACT AGT AC
Round2-pSAM	CGC GCC AAG CAG AAG ACG GCA TAC G
LIB_AdaptT_4	TTC CCT ACA CGA CGC TCT TCC GAT CTT TTC CNN
LIB_AdaptB_4	GGA AAA GAT CGG AAG AGC GTC GTG TAG GGA A

Table 7: Sequence of primers employed throughout the study.

Pyrimidine Metabolism	Lipopolysaccharide biosynthesis	Peptidoglycan biosynthesis	Pantothenate and CoA biosynthesis	Nicotinate and nicotinamide metabolism
PGN_0207	PGN_0206	PGN_0622	PGN_1487	PGN_0207
PGN_0646	PGN_0544	PGN_0623	PGN_1515	PGN_0670
PGN_0997	PGN_0679	PGN_0625	PGN_1593	PGN_1441
PGN_0894	PGN_2019	PGN_0627	PGN_1784	PGN_2006
PGN_1001	PGN_2020	PGN_0760		PGN_2007
PGN_1022	PGN_2086	PGN_0817		
PGN_1447		PGN_1130		
PGN_1570		PGN_1153		
PGN_1571				
PGN_1771				
PGN_1789				
PGN_1833				
PGN_1841				
PGN_2062				

Table 8: Pathways enriched among inherently essential *P. gingivalis* genes.

3.4. Discussion

A comparison of two independently generated TnSeq libraries for *P. gingivalis* ATCC 33277 suggests that 281 common genes are essential for *in vitro* growth in two complex media. This minimal *P. gingivalis* genome is rich in genes performing critical physiological processes, particularly pyrimidine metabolism as well as lipopolysaccharide, peptidoglycan, and coenzyme A biosynthesis. Those genes that encode established virulence factors, such as gingipains [101], fimbriae [314], capsule [105], and RagB [315], are underrepresented in this overlapping gene set, probably because there is no immune or competing microbial challenge to be met. In other words, they are likely to be conditionally essential rather than inherently essential. Hence it could be reasoned that, while targeting *P. gingivalis* components that drive pathogenesis *in vivo* is a valid approach, the rather more mundane set of essential genes identified herein may prove to be more efficacious in controlling *P. gingivalis* infections. It should be noted that the 'essential' gene set can change depending on the growth conditions. Some genes will likely be required in all conditions (e.g. *rpoN* and *lepB*, identified herein) but the importance of others will depend on the nutritional content of the medium, i.e. conditionally essential genes. In the long-term, a comparison of inherently essential genes and genes essential in multiple conditions will facilitate optimization of potential therapeutic targets.

In addition to the elucidation of genes grouped into metabolic pathways, there are other essential genes that are interesting at the individual level. The *rpoN* gene (PGN_1202, 33277; PG1105, W83 orthologue; PGTDC60_1103, TDC60 orthologue), for example, encodes the alternative sigma factor, σ^{54}. *Bacteroides* spp. are rich in extracytoplasmic sigma factors compared with other *Bacteroidetes* [316]. These sigma factors form part of the RNA polymerase holoenzyme and control transcriptional specificity. σ^{54} is commonly used for large-scale transcriptional changes by binding to different and more highly

conserved consensus sequences than those for σ^{70} [317]. Furthermore, although there are multiple members of the σ^{70} family – and the σ^{70} factor *rpoD* (PGN_0638; PG0594; PGTDC60_1719) is also essential – there is only one member of σ^{54}. Interestingly, *rpoN* is not typically an essential gene in most systems. The only described instance where *rpoN* has been shown to be essential in the soil bacterium, *Myxococcus xanthus* [318]. Why the *P. gingivalis rpoN* gene is essential for *in vitro* growth remains to be determined.

The heat-shock protein 60 homolog, *groEL* (PGN_1452; PG0520; PGTDC_1639), is an emergent *P. gingivalis* virulence factor that has been proposed to promote cytokine production and inflammation, to enhance osteoclastogenesis, to aid in the vascularization of tumors and, perhaps, to play a role in atherosclerosis [319-322]. Although PGN_1452 is not itself essential, the *groEL* co-chaperone, *groES* (PGN_1451; PG0520; PGTDC60_1640) is required for growth in complex media. Another heat-shock protein family member, *dnaJ* (PGN_1716; PG1776; PGTDC60_1360), and the gene encoding the DnaK repressor (PGN_0516; PG1597; PGTDC60_0704) are further common essential genes.

There are several proteolytic genes common to Library 1 and Library 2. These are genes encoding: signal peptidase I (*lepB*; PGN_1946; PG1598; PGTDC60_0703); signal peptidase II (*lspA*; PGN_0515; PG2001; PGTDC60_0274); as well as an aminoacyl-histidine dipeptidase (PGN_0250; PG0137; PGTDC60_0414); and a leucine aminopeptidase (PGN_0202; PG2157; PGTDC60_1640) that is related to the M28 group of metalloproteinases. *P. gingivalis* contains only a single copy each of *lepB* and *lspA*, which are required for protein translocation across cell membranes, consistent with the finding that these signal peptidases form part of the core genome. Additionally, the

majority of Sec-related protein export genes were also essential (*secY*, *secE*, *secG*, *secD/F*, *yajC*, *SecA*, *SRP54/ffh*, and *ftsY* but not *yidC*).

Multiple genes that control bacterial shape, including those encoding RodA (PGN_0626; PG703; PGTDC60_0579 and PGN_0870; PG1392; PGTDC60_2072), as well as MreB (PGN_0866; PG1396; PGTDC60_2068), are also common inherently essential genes. Interestingly, *mreB* is not usually an essential gene for most rod-shaped bacteria. Perhaps the essentiality of *mreB* in *P. gingivalis* in laboratory media suggests a need to maintain a rod shape to keep the surface area-to-volume ratio lower than that for a coccus.

Upon interrogation of the Database of Essential Genes, it is clear that some *P. gingivalis* ATCC 33277 genes have common essential homologs whereas others are unique (*n* = 54; 19%) to this bacterium. Eighty-three (30%) genes have ≤10 essential homologs, while 11 (*n* = 4%) have ≥50 homologs. Although it must be acknowledged that the current database of known essential prokaryotic genes is limited to 36 bacterial species other than *P. gingivalis* – because of the recent emergence of TnSeq technology – the combination of common and unique required genes suggests that, in the long term, it may be possible to generate both broad-spectrum and *P. gingivalis*-specific therapeutic targets for the control of bacterial infections.

Differential phenotypes are sometimes reported for the same bacterial strains. Although this can be partly explained, for example, by different model systems or the propagation of relevant mutant genes within laboratory collections, the comparison of Library 1 and Library 2 raises another possibility. Despite the fact that both libraries are *P. gingivalis* ATCC 33277-specific, approximately half of the genes identified in Library 1 were not common to Library 2. Considering that both libraries lack transposon insertion hot spots and contain large mutant numbers (54,000 and 80,000 colonies, respectively), the major difference between the experimental setups is the composition of

the complex growth media employed (Table 6). As different nutritional and other needs may be met by each of these complex media, this may be sufficient to induce considerable differences in the adaptive response to such growth conditions, reflected in the different profiles of essential genes. Performing similar analyses on other bacterial pathogens to distinguish between inherently essential and conditionally essential genes may reveal additional targets for new therapeutics.

In summary, we have identified 281 genes that are essential for *in vitro* survival of the periodontal pathogen *P. gingivalis* as defined by two independently generated TnSeq mutant libraries. It is expected, following validation of these genes by determining the growth characteristics of essential gene deletion mutants and their complements, that some of these required genes will represent novel therapeutic targets for the efficient control of *P. gingivalis*.

CHAPTER 4

IDENTIFICATION OF GENES ESSENTIAL FOR *PORPHYROMONAS GINGIVALIS* SURVIVAL UNDER THE CONDITION OF CIGARETTE SMOKE INDUCED STRESS

4.1. Introduction

There are >1.3 billion smokers in the world (177). Smoking alone accounts for most cases of periodontitis in adults in developed nations, like Scandinavia, New Zealand and the U.S. [323-326]. Smokers exhibit reduced clinical signs of inflammation. In smokers, host- and bacterial-derived tissue degrading proteinases enhance periodontal destruction [327-334]. Results of several cross-sectional and longitudinal studies showed smoking as a major environmental risk factor associated with the development of extensive and severe periodontal diseases [159, 198, 201, 335, 336]. Smoking results in periodontal destruction in a dose-dependent manner [337, 338]. Probing pocket depths and tooth mobility scores were highest among smokers having periodontitis [339, 340]. Oxygen tensions in periodontal pockets are lower in smokers compared to non-smokers [171]. In smokers, there is a chronic reduction of blood flow, altered neutrophil function, altered cytokine and growth factor production, inhibition of fibroblast growth and attachment, decreased collagen production and vascularity in the oral cavity, but increased levels of free radicals and lipid peroxidation in periodontal tissues, and alteration in osteoclast activity [339, 341]. All these changes affect the pathogenesis of periodontal diseases and healing of the periodontium after periodontal treatment in smokers. Thus, the local oral environment in smokers is different from that of non-smokers. These changes in the oral environment might exert selective pressure on the oral biofilms and in turn, lead to the selection of a specific group of microorganisms.

Many recent studies have established that cigarette smoke considerably affects subgingival bacterial ecology. Smokers demonstrate a pathogen-rich, commensal-poor, anaerobic, and dysbiotic oral microbiome, even in periodontally healthy individuals [342]. Despite the conflicting reports regarding the influence of smoking on the oral microbiome, the microbial profile of smokers having periodontitis is distinct from that of non-smokers [200, 342-344]. These studies suggest that there exists an association between smoking and subgingival dysbiosis. Dysbiosis in smokers plays a significant role in the increased severity of periodontitis [345].

In a study by Coretti *et al.,* analysis of the putative biological functions of subgingival microbial communities in smokers having periodontitis, non-smokers with periodontitis and healthy individuals was performed [345]. In the microbiota of healthy subjects, enzymes for biosynthesis and biodegradation of secondary metabolites, fatty acid metabolism, and amino acid biosynthesis were upregulated. In smokers having periodontitis, there was enrichment of genes encoding peptidases, enzymes for lipopolysaccharide biosynthesis, and proteins involved in cell motility and secretions compared to control subjects. Enrichment of such genes, suggests that there is enhanced virulence of subgingival microbiota in smokers compared to the healthy individuals. Further, in smokers having periodontitis, enzymes involved in cell motility and secretion, homologous recombination, peptidase, and DNA replication were over-represented in the subgingival microbiota compared to non-smokers with periodontitis. In a study done by Bagaitkar *et al.*, it has been shown that CSE significantly influences the expression of 6.8% of the *P. gingivalis* genome, as determined by whole genome arrays [180]. These included genes regulating capsule and fimbriae, corresponding to the reduced pro-inflammatory potential of *P. gingivalis*; oxidative stress and DNA repair. *P. gingivalis*

adapts to the environmental stress presented by CSE by altering the expression of several genes and outer membrane proteins [180].

Despite information regarding various virulence factors of *P. gingivalis* and their host interactions at the phenotypic level, the genes essential for the survival of *P. gingivalis* in smokers have not been identified. In this study, we plan to identify the genes of *P. gingivalis* ATCC 33277 that are essential in cigarette smoke conditioned GAM using massively parallel sequencing technology [103, 311, 346, 347]. Due to the quantitative nature of massively parallel sequencing, the fitness of a gene knockout can be quantitatively determined and is a direct measure of the growth rate of the bacterial clone containing it. The obtained information, in turn, might help us to gain information regarding the metabolic pathways and adaptation mechanisms. Hence, the study of genes regulating various virulence factors under the influence of cigarette smoke extract is of interest in this project. Wwe are interested in identifying the genes necessary for the survival of *P. gingivalis* in cigarette smoke-exposed GAM (Gifu anaerobic media) through the construction and analysis of transposon mutant libraries. We further hypothesized that we could verify conditional essentiality by the establishment of reduced relative growth of a subset of specific bacterial mutants when competed against the parent *P. gingivalis* strain under conditions of CSE-induced stress.

4.2. Materials and Methods

Materials

P. gingivalis ATCC 33277 Tnseq library generated in Chapter 3 was cultured in Gifu anaerobe medium (GAM; Nissui Pharmaceutical, Tokyo, Japan), liquid GAM conditioned with the cigarette smoke extract or on GAM-blood agar. Sheep blood was obtained from Lampire Biological Laboratories (Pipersville, PA). Gentamicin, erythromycin, tetracycline,

ampicillin, dichloromethane, sodium sulfate and analytical nicotine standards were from Sigma-Aldrich (St. Louis, MO). All primers were from Biosynthesis Inc. (Lewiston, TX). Lonza flash gels came from Lonza (Rockland, ME). Wizard SV and PCR clean-up kit were from Promega (Madison, WI). T4 DNA Ligase and buffer; *Mme*I; and TAE buffer came from New England Biolabs (Ipswich, MA), while HiFi Hotstart Ready mix was from KAPA Biosystems (Wilmington, MA) and the polymerase chain reaction (PCR) SuperMix came from Invitrogen (Carlsbad, CA). Fast digest restriction enzymes, T4 DNA ligase, ready to use x-gal and plasmid isolation kit came from Thermoscientific. High-efficiency transformation neb alpha cells came from BioEngland. DNA yields were quantified using a NanoDrop ND-1000 spectrophotometer (Wilmington, DE). We obtained *P. gingivalis* strains with mutations in PGN_1524, PGN_1474, PGN_0770, PGN_0714, PGN_0491, PGN_0287, or PGN_0088 from Dr. Richard J Lamont (University of Louisville). We obtained *P. gingivalis* PGN_1200, PGN_1444, or PGN_0898 mutant strains from Dr. Jan S Potempa (University of Louisville).

Conditioning GAM with cigarette smoke extract (CSE)

GAM was conditioned using standard reference cigarettes (2R1, Kentucky Tobacco Research, and Development Center). By using a three-way stopcock and a syringe, GAM-CSE was prepared by drawing cigarette smoke through 50 ml of the medium, with 35 ml drags performed every 20 seconds. GAM-CSE was then filtered (0.2 mm). Nicotine content was determined by gas-liquid chromatography and adjusted to pH 7.2 and 1000 ng/ml nicotine concentration. *P. gingivalis* ATCC 33277 TnSeq library was grown in their respective control and cigarette smoke extract conditioned media under anaerobic conditions (80% N_2, 10% H_2, 10% CO_2) at 37°C. For all experiments, bacteria were grown either in control or CSE conditioned media, and bacterial cells were harvested at mid to late log phase (*P. gingivalis*- $O.D._{600}$ nm =1.0, corresponding to 10^9 cells per ml)

Identification of essential genes for *P. gingivalis* survival in GAM-CSE:

a) Library passage in GAM and GAM-CSE:

P. gingivalis ATCC 33277 Tnseq mutant library aliquots described in chapter 3 were grown in GAM (Control), and GAM-CSE containing 5 µg/ml erythromycin and 50 µg/ml gentamycin, as *P. gingivalis* is naturally resistant to gentamycin and the transposon system that is used to create mutant library has erythromycin resistance. After the cultures reached mid-late log phase, cultures were passaged twice in GAM and GAM-CSE.

b) Preparation of DNA for TnSeq analysis:

Genomic DNA was isolated using Wizard DNA isolation kit and purified using Wizard purification kit, as per the manufacturer's instructions. DNA yields were quantified using a NanoDrop ND-1000 spectrophotometer. After DNA isolation, 2 different double-stranded DNA (dsDNA) adapter molecules were created, by using primers LIB_AdaptT_3 and LIB_AdaptB_3 for CSE model and LIB_AdaptT_4 and LIB_AdaptB_4 for control (Table 7). The primers were combined and heated at 95°C for 5 min in a water bath and then allowed to cool to room temperature. Then, they were aliquoted and stored at -20°C. The genomic DNA obtained from the previous step was set for digestion with a Mme1 restriction enzyme for 1 hour at 37°C followed by for 20 min at 80°C. Then, the obtained reactions were purified using Wizard purification kit. This purified digested DNA was then run on 1% agarose gel and the band corresponding to approximately 1500 bp (as it corresponds to the additive length of both transposon and the flanking sequences) was extracted using Wizard gel extraction kit. This Mme1 digested DNA was then ligated to barcoded adapter molecules created using T4 DNA ligase incubated for 16 h at 16°C followed by heat inactivation for 10 min at 65°C and purified using Wizard purification kit. This ligated purified product further amplified by performing PCR using HiFi Hotstart

SuperMix with LIB_PCR_5 and LIB_PCR_3 primers (Table 7) using the following PCR cycle : 98°C for 3 min, 98°C for 20 sec,60°C for 1 min,72°C for 1 min for 18x cycles and 72°C for 4 min. The obtained PCR product was purified using Wizard purification kit and to verify the 125 bp band Lonza flash gel system was used. Further, the obtained samples were quantified using NanoDrop ND-1000 spectrophotometer. Samples were sent to the University of Michigan for sequencing using massively parallel sequencing technology, Illumina HiSeq2000 platform for quantitative assessment of individual mutants in the library by sequencing the transposon-genomic junction.

c) TnSeq Data Analysis:

CLC Bioinformatics Workbench 5.5.1 software was used to analyze the TnSeq results. In the first step, reads having a quality score <0.05 or of <15 nucleotides were discarded. The negative selection method was employed in order to identify the conditional essential genes in *P. gingivalis*. The remaining reads were aligned to the annotated whole genome of *P. gingivalis* ATCC 33277 strain. In each library, 8-14 million reads were identified with ,on average, 3900 – 6900 genes/read with 52-81% of genes having at least one hit. A total of 258 conditional essential genes in GAM-CSE model were identified using the criteria of >50 fold change, p<0.01. DAVID database (*david.niaid.nih.gov*) and KEGG *(*www.genome.jp/kegg) *in silico* analyses were used to identify the functions and interrelationships of these identified conditional essential genes.

e) Construction of bacterial strains and plasmids:

P. gingivalis PGN_0388 deletion mutant was generated by homologous recombination, as previously described [348]. In brief, two 1-kb flanking regions on either side of the PGN_0388 were amplified from genomic DNA by PCR with primer pairs UF, UR and DF and DR as shown in Table 12. The PCR product was cloned into plasmid pUC19 with an

inserted erythromycin-resistance (*erm*) cassette (*ermF-ermAM*) from p1403CeB-H. Correct placement and orientation of the DNA segments in the resulting pHBG3 plasmid were confirmed by sequencing. The resulting suicide plasmid pHBG3 was electroporated into electrocompetent *P. gingivalis* ATCC 33277 cells for generating the PGN_0388 mutant, as previously described [349]. Resultant clones were selected on erythromycin plates, and double-crossover genomic recombination was confirmed by DNA sequencing of the manipulated region.

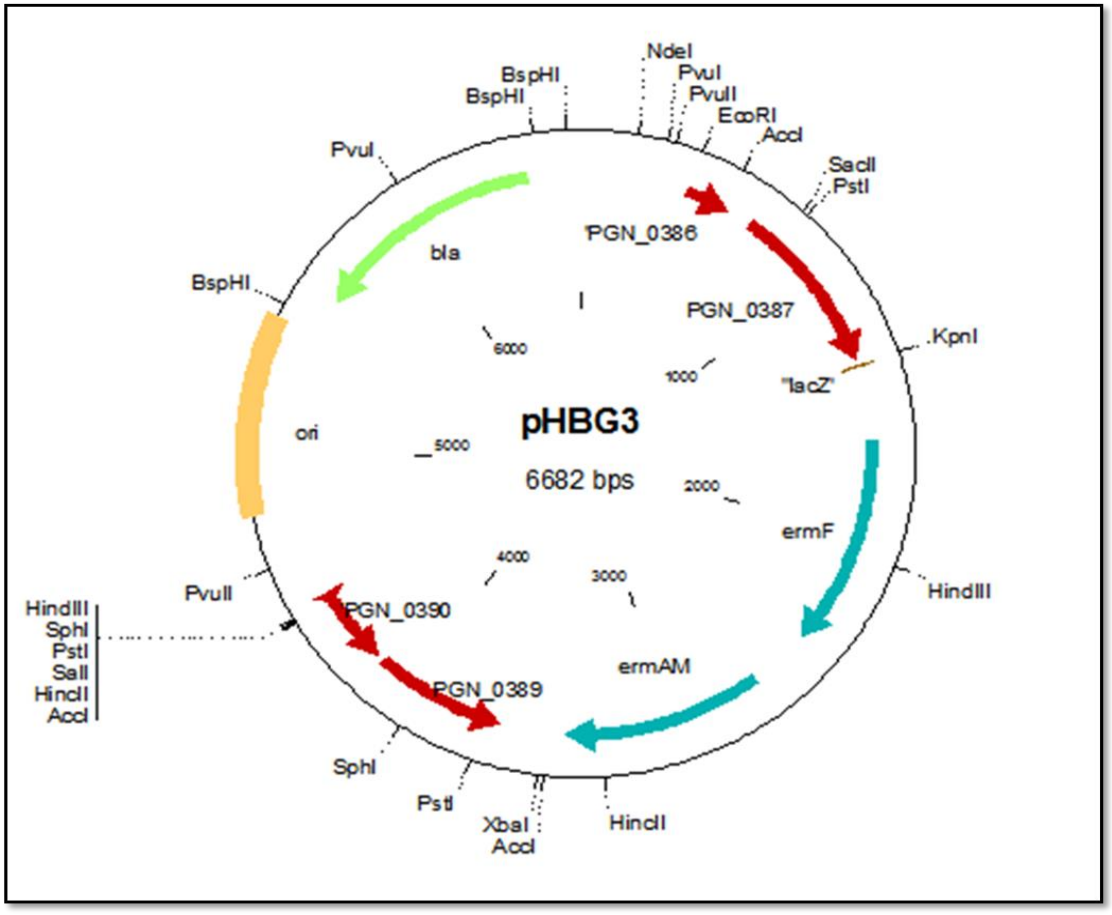

Figure 5: The pHBG3 plasmid having *ori*- the origin of replication, *bla* – beta lactamase gene, *ermAm-ermF* – erythromycin gene cassette and PGN_0388 gene flanking regions.

f) Competition Assays

To confirm that the selected mutant strains are specifically outcompeted by the wild-type strain in GAM-CSE, competition assays were carried out in *in vitro*. *P. gingivalis* ATCC 33277 and mutant strains were co-cultured in 1:1 ratio in triplicates for two passages in GAM, followed by one passage in GAM-CSE having 1000 ng/ml nicotine equivalent. When the $O.D._{600}$ of cultures reached mid-late log phase, the GAM-CSE culture was plated on GAM-blood agar plates with or without antibiotic (5 µg/ml of erythromycin and 5 µg/ml of tetracycline for PGN_1200 and PGN_1444). The GAM-CSE culture was spread on the plates in triplicate and placed in the anaerobic chamber. After incubation for seven days, colony forming units (CFU) were determined. The ratio of the CFU on GAM-blood agar plates with or without antibiotics was determined. The mutant strain was considered as outcompeted by the wild-type if ≥ 2:1 ratio between wild-type and the mutant strain was observed.

4.3. Results

The complete database of 258 identified conditional essential genes (50+ fold change, $p<0.01$), in GAM-CSE is provided in Table 10. The identified genes represent approximately 8% of the *P. gingivalis* ATCC 33277 genome and are presented in Figure 6 using the CGViewer software. KEGG analysis (www.genome.jp/kegg) was employed to functionally classify the identified gene set, as noted in Table 9. Out of 258 identified essential genes, 22 were annotated as metabolic; 1 as mismatch repair; 1 as aminoacyl-tRNA biosynthesis; and two as ABC transporters. The metabolic-annotated genes are related to pyrimidine metabolism, nicotinate and nicotinamide metabolism, purine metabolism, pentose phosphate metabolism, nitrogen metabolism, seleno-compound metabolism, alanine aspartate and glutamate metabolism, cysteine, and methionine metabolism. Similar functional groupings were obtained using the DAVID database

(https://david.ncifcrf.gov/). Functional relationships between the identified conditional essential genes were determined using KEGG analysis as shown in Figures 7a-h.

To validate the Tn-seq results, we are interested in looking at the top genes according to fold change, and also the genes of biological significance. PGN_ 0407 (hypothetical gene), PGN_ 0025 (spoU;rRNA methylase family protein), PGN_1494 (Oxygen-independent coporphyrinogen III oxidase), PGN_ 0388 (Thiol peroxidase), PGN_0604 (Ferritin) are the top hits according to the fold change. We were able to generate the PGN_0388 mutant using site-directed mutagenesis. The other genes of biological significance are PGN_1524 encoding tyrosine kinase, PGN_1474 (*luxS*) encoding S-ribosyl homocysteine lyase, PGN_1200 encoding putative ATPase,PGN_1444 (*carA*) encoding carbamoyl-phosphate synthase small chain, PGN_0770 (*rnz*) encoding ribonuclease Z, PGN_0714 encoding probable pyrazinamidase/ nicotinamidase, PGN_0491 encoding probable phosphotyrosine protein phosphatase, PGN_0287 (*mfa1*) encoding minor fimbrium subunit Mfa1 , PGN_0088 encoding putative transcriptional regulator. These genes have a fold change of >400 in GAM-CSE model except for PGN_1444 and PGN_0770, which have a fold change of 172 and 108 respectively. In the competition assays, all the mutants except PGN_1444 were outcompeted by the wild type *P. gingivalis* ATCC 33277 strain. PGN_1753 was used as a control, and its growth not compromised.

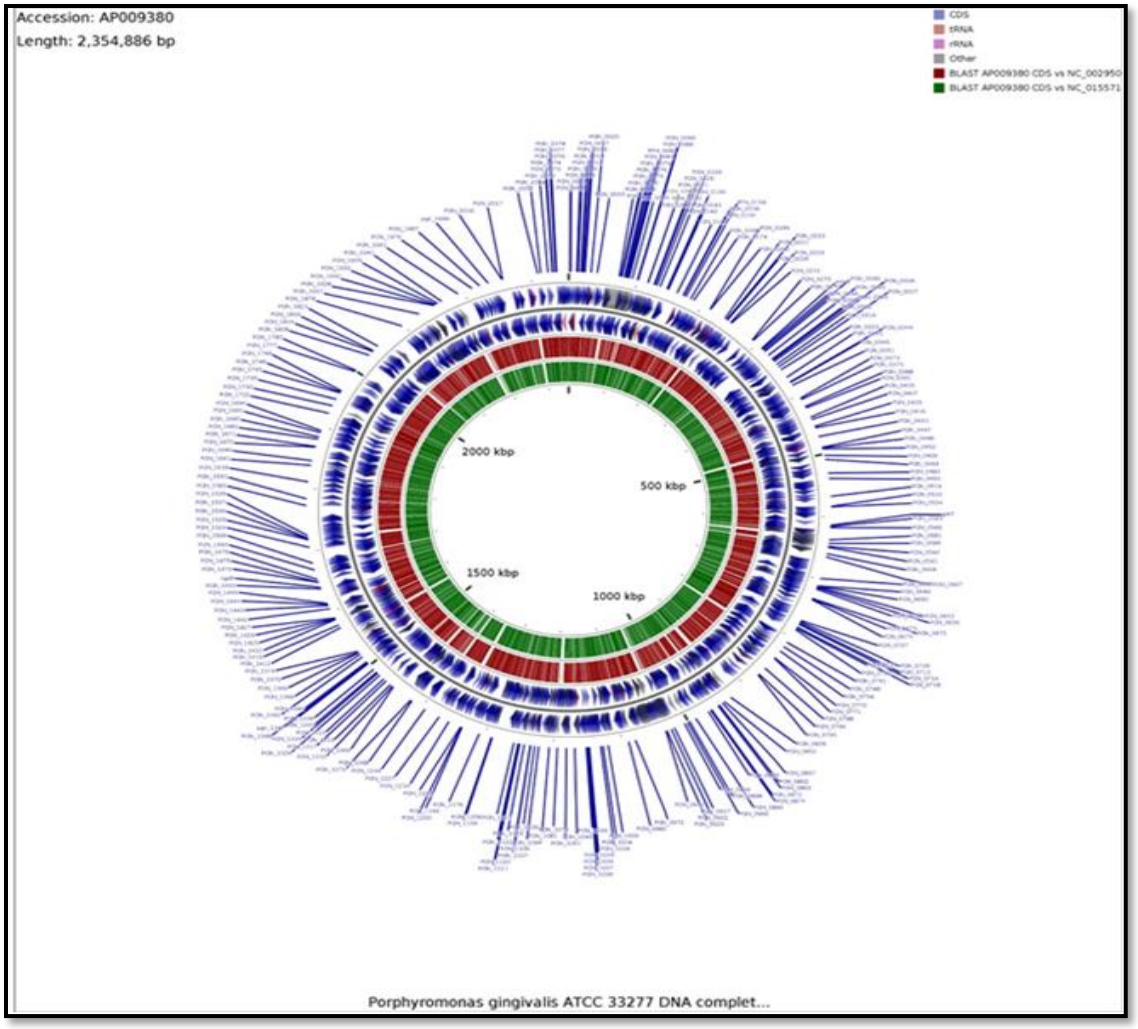

Figure 6: Gene map depicting genes essential for the survival of *P. gingivalis* ATCC in GAM-CSE model (Gifu anaerobic medium conditioned with cigarette smoke extract). Essential genes were mapped to *P. gingivalis* ATCC 33277 genome using CG Viewer. The blue arrows show the location of the 257 essential genes in GAM-CSE model within the *P. gingivalis* 33277 chromosome. The adjacent two rings depict gene orientation(outer and inner blue arrows represent the positive and negative strands, respectively). The next two rings (red and green) represent the blast hits in relation to *P. gingivalis* TDC60 and W 83 strains, respectively.

Carbohydrate Metabolism	9/86 (10%)
Nitrogen Metabolism	1/5 (20%)
Pyrimidine Metabolism	5/30 (16%)
Purine Metabolism	2/35 (5%)
Amino Acid Metabolism	9/74 (12%)
Nicotinate and Nicotinamide Metabolism	5/13 (38%)
Thiamine Metabolism	1/7 (14%)
Porphyrin Metabolism	2/30 (6%)
Folate Biosynthesis	1/9 (11%)
Aminoacyl tRNA Biosynthesis	1/4 (12%)
Mismatch Repair	1/9 (11%)
Signal Transduction	2/9 (22%)
Aminobenzoate Degradation	1/2 (50%)

Table 9: Functional classification of conditionally essential genes of *P. gingivalis* in GAM-CSE based on KEGG analysis.

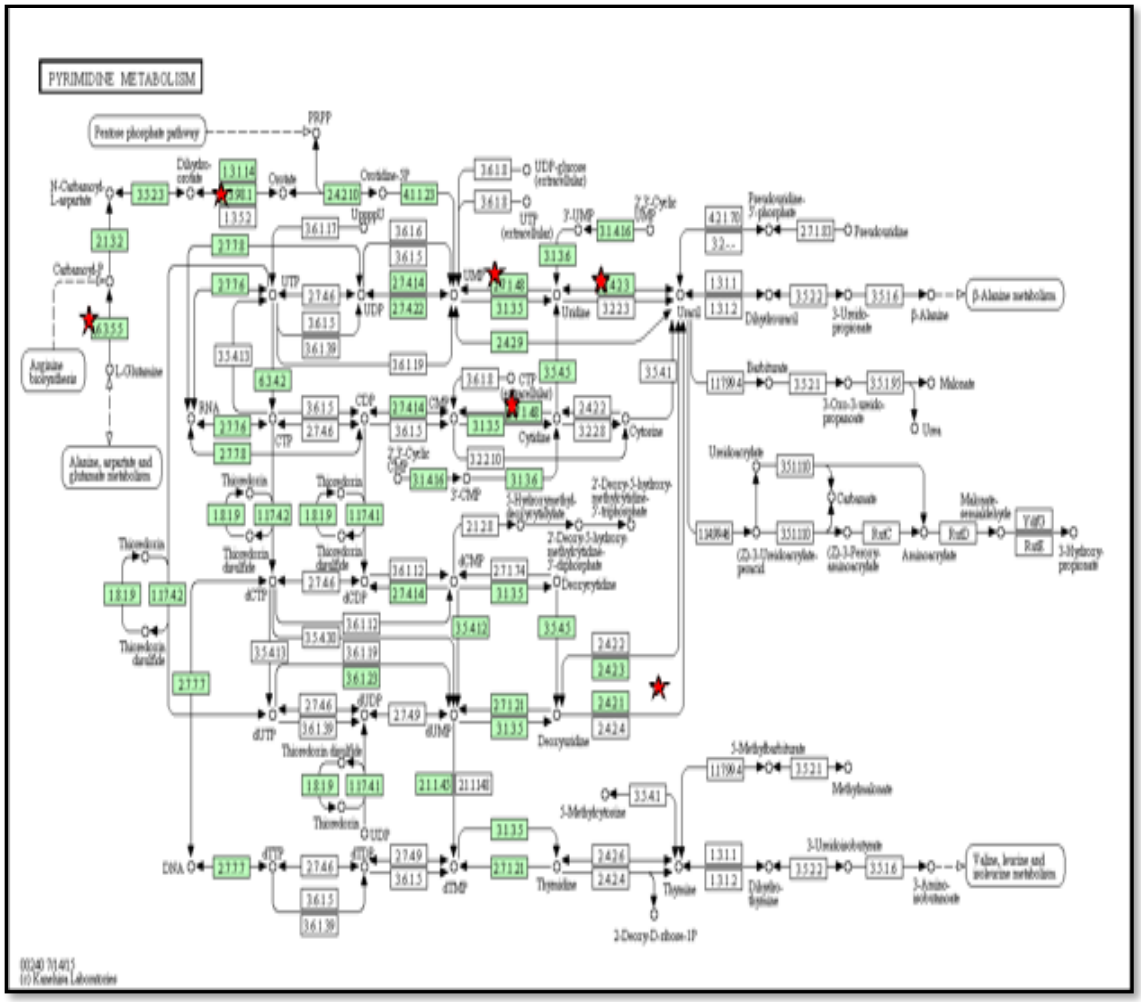

Figure 7a: Pyrimidine Metabolism (pathway enriched among conditional essential *P. gingivalis* genes in GAM-CSE). KEGG pathways (www.genome.jp/kegg) are presented with the proteins catalyzing each step shown by their enzyme commission number and substrates/products. Green shading indicates the presence of homologs in *P. gingivalis*. The red star denotes essential genes. The PGN assignments of the essential metabolic genes are provided in Table 11.

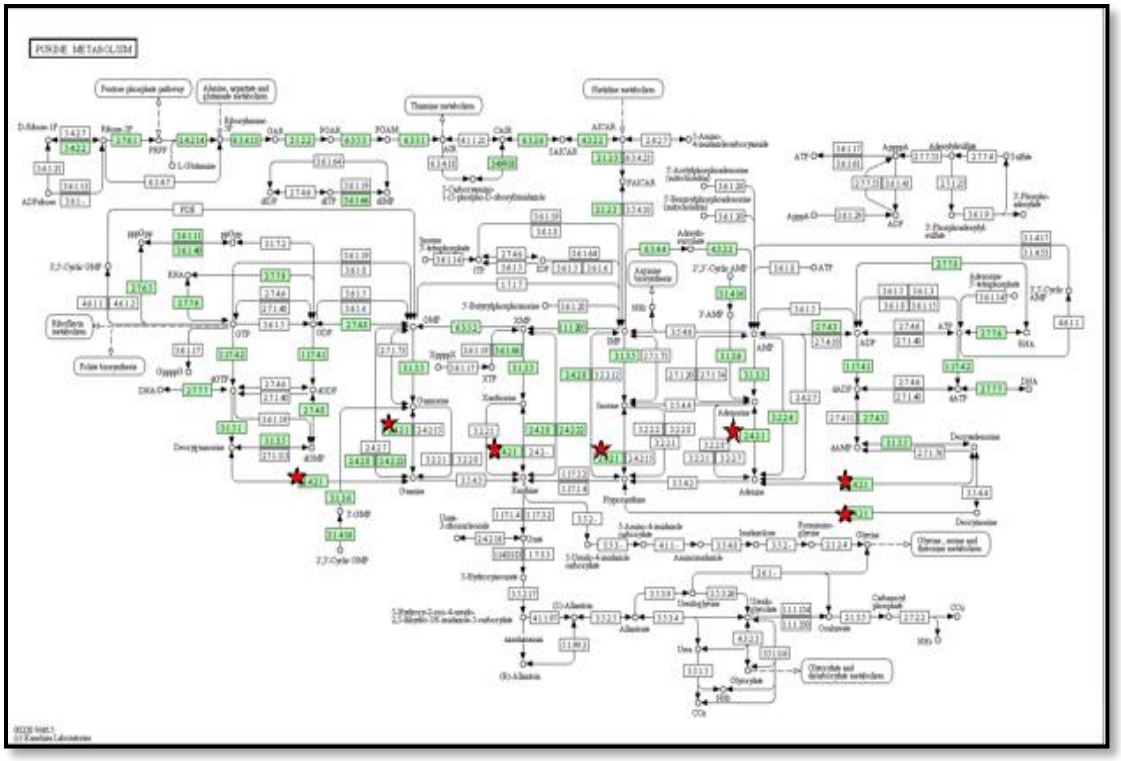

Figure 7b: Purine Metabolism (pathway enriched among conditional essential *P. gingivalis* genes in GAM-CSE). KEGG pathways (www.genome.jp/kegg) are presented with the proteins catalyzing each step shown by their enzyme commission number and substrates/products. Green shading indicates the presence of homologs in *P. gingivalis*. The red star denotes essential genes. The PGN assignments of the essential metabolic genes are provided in Table 11.

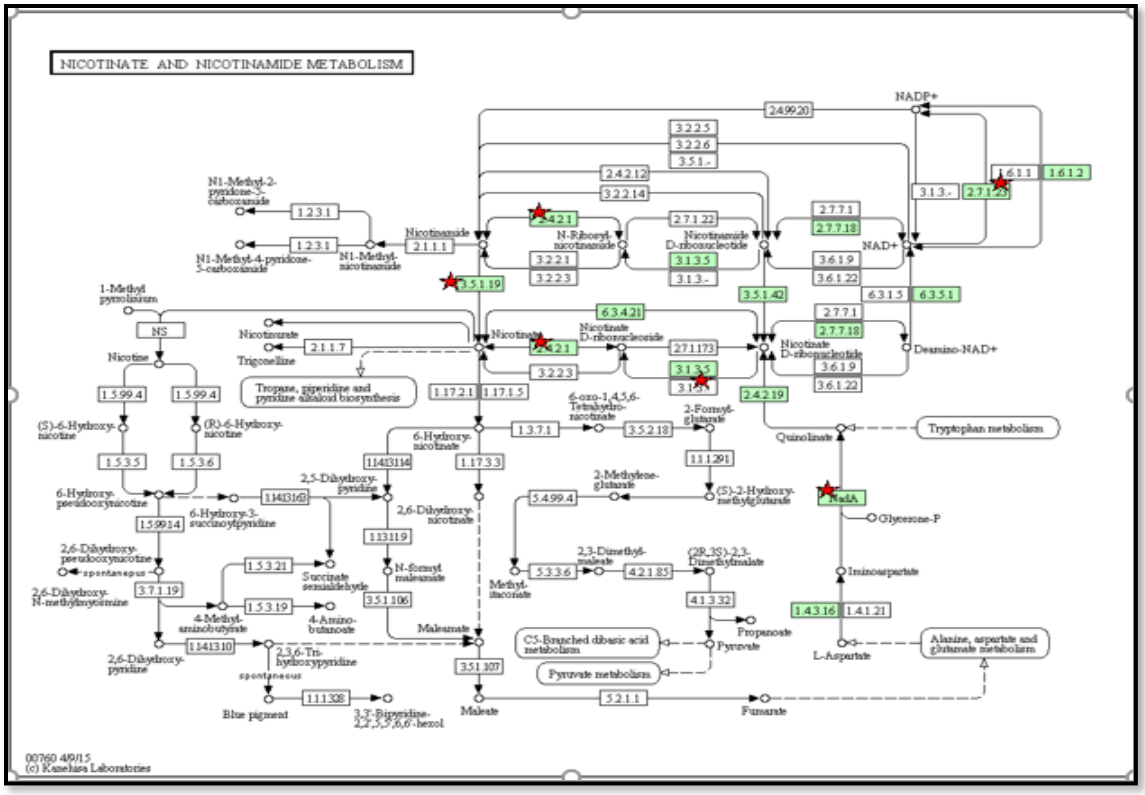

Figure 7c: Nicotinate and Nicotinamide Metabolism (pathway enriched among conditional essential *P. gingivalis* genes in GAM-CSE). KEGG pathways (www.genome.jp/kegg) are presented with the proteins catalyzing each step shown by their enzyme commission number and substrates/products. Green shading indicates the presence of homologs in *P. gingivalis*. The red star denotes essential genes. The PGN assignments of the essential metabolic genes are provided in Table 11.

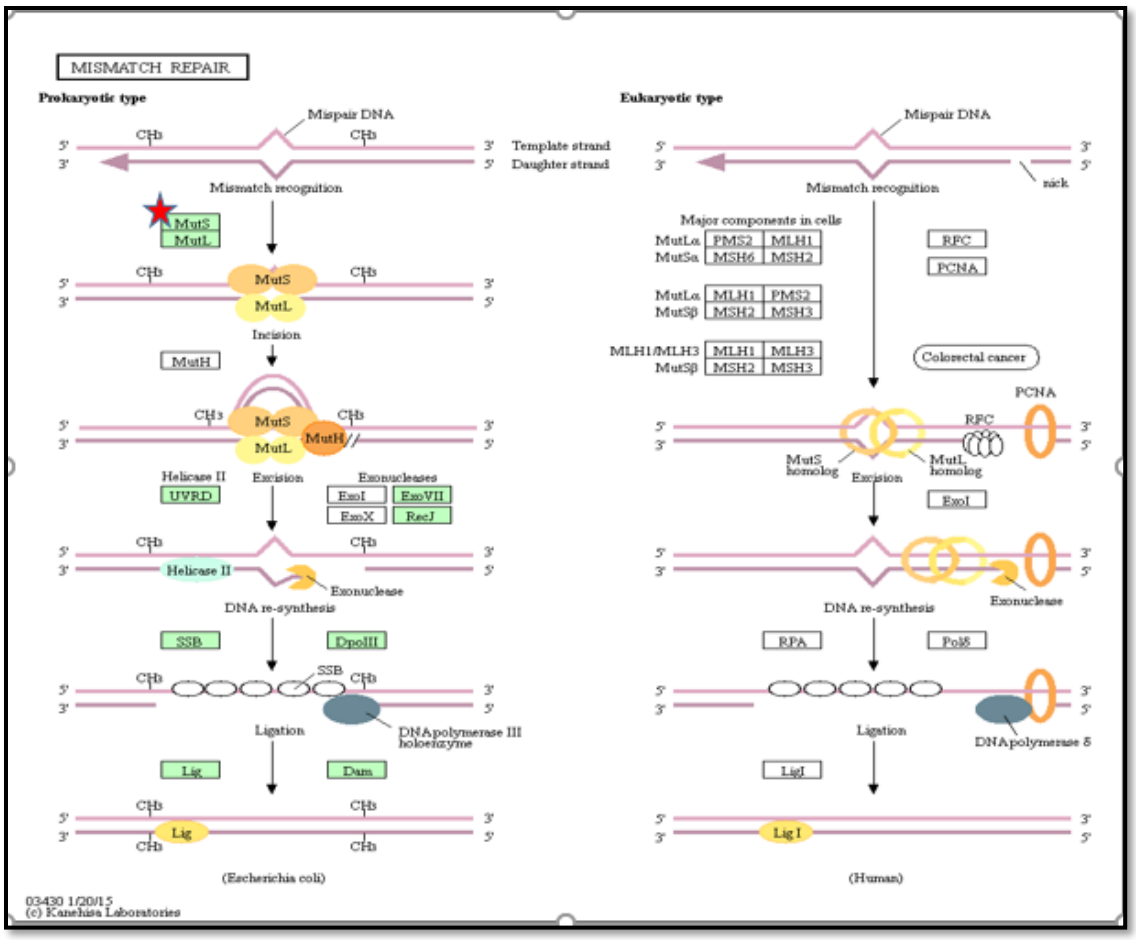

Figure 7d: Mismatch Repair (pathway enriched among conditional essential *P. gingivalis* genes in GAM-CSE). KEGG pathways (www.genome.jp/kegg) are presented with the proteins catalyzing each step shown by their enzyme commission number and substrates/products. Green shading indicates the presence of homologs in *P. gingivalis*. The red star denotes essential genes. The PGN assignments of the essential metabolic genes are provided in Table 11.

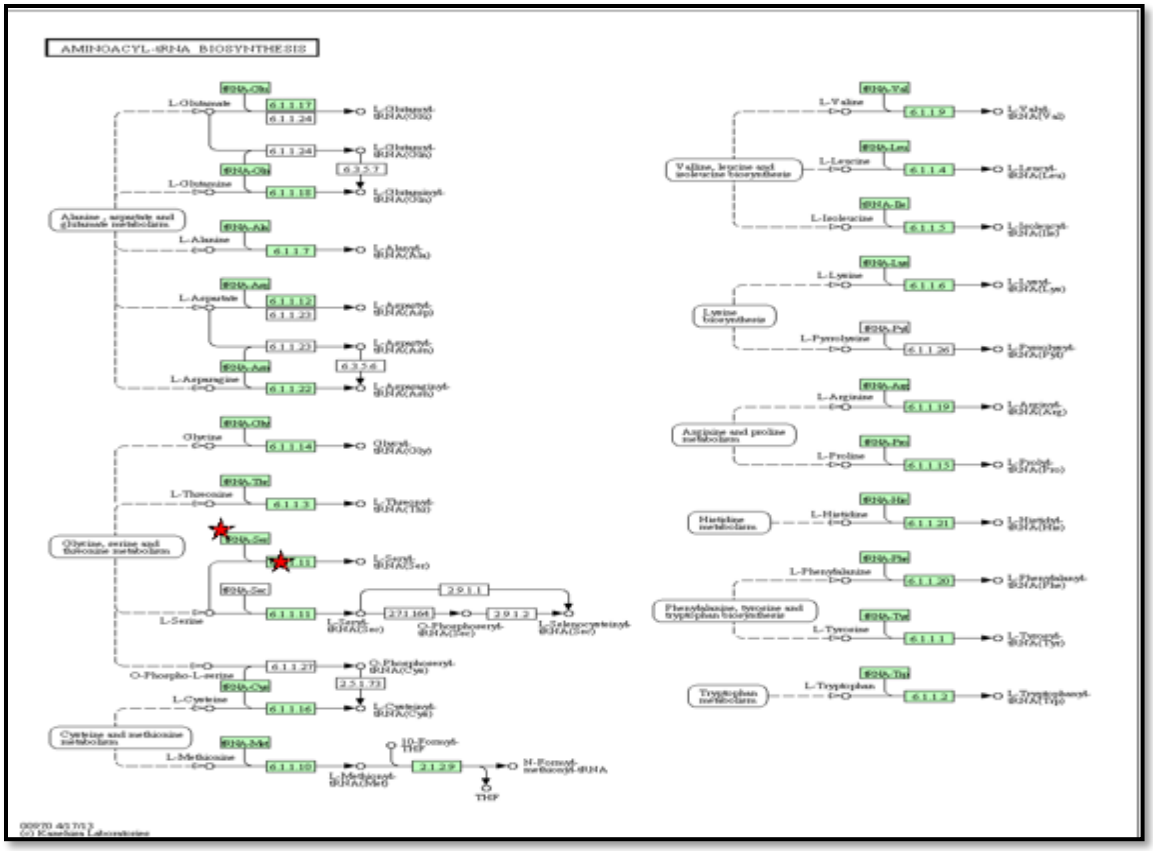

Figure 7e: Aminoacyl tRNA Biosynthesis (pathway enriched among conditional essential *P. gingivalis* genes in GAM-CSE). KEGG pathways (www.genome.jp/kegg) are presented with the proteins catalyzing each step shown by their enzyme commission number and substrates/products. Green shading indicates the presence of homologs in *P. gingivalis*. The red star denotes essential genes. The PGN assignments of the essential metabolic genes are provided in Table 11.

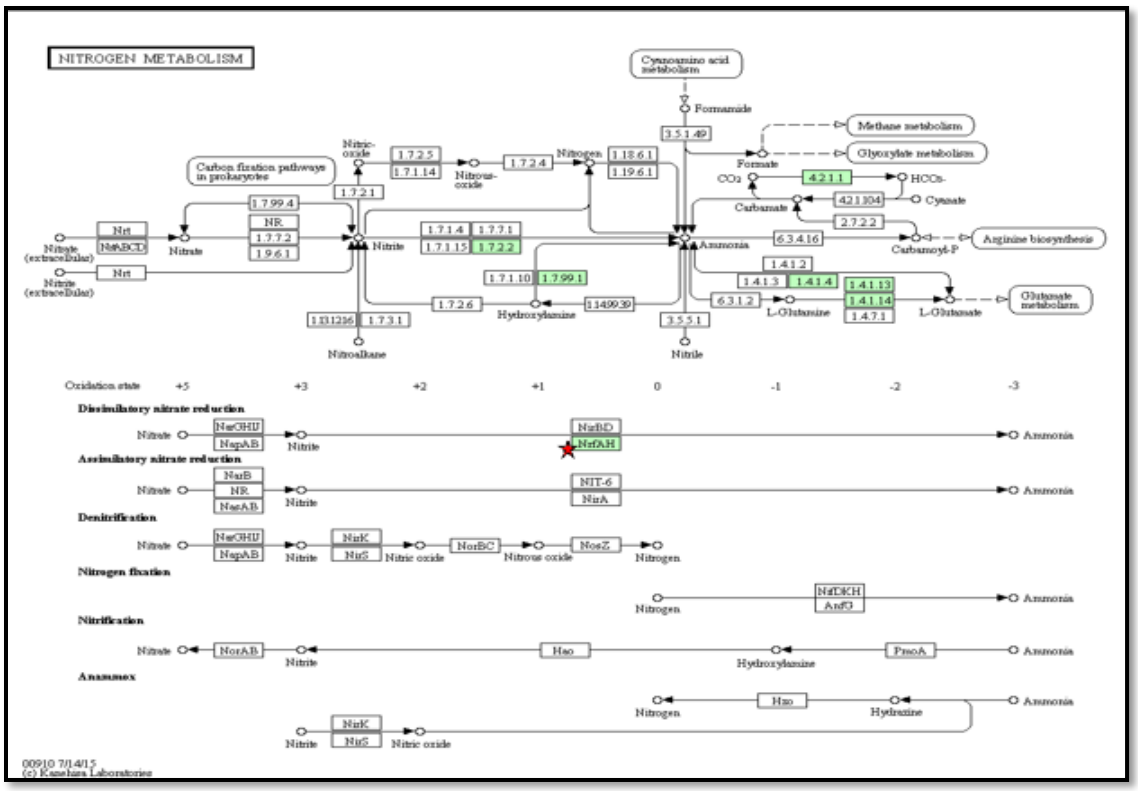

Figure 7f: Nitrogen Metabolism (pathway enriched among conditional essential *P. gingivalis* genes in GAM-CSE). KEGG pathways (www.genome.jp/kegg) are presented with the proteins catalyzing each step shown by their enzyme commission number and substrates/products. Green shading indicates the presence of homologs in *P. gingivalis*. The red star denotes essential genes. The PGN assignments of the essential metabolic genes are provided in Table 11.

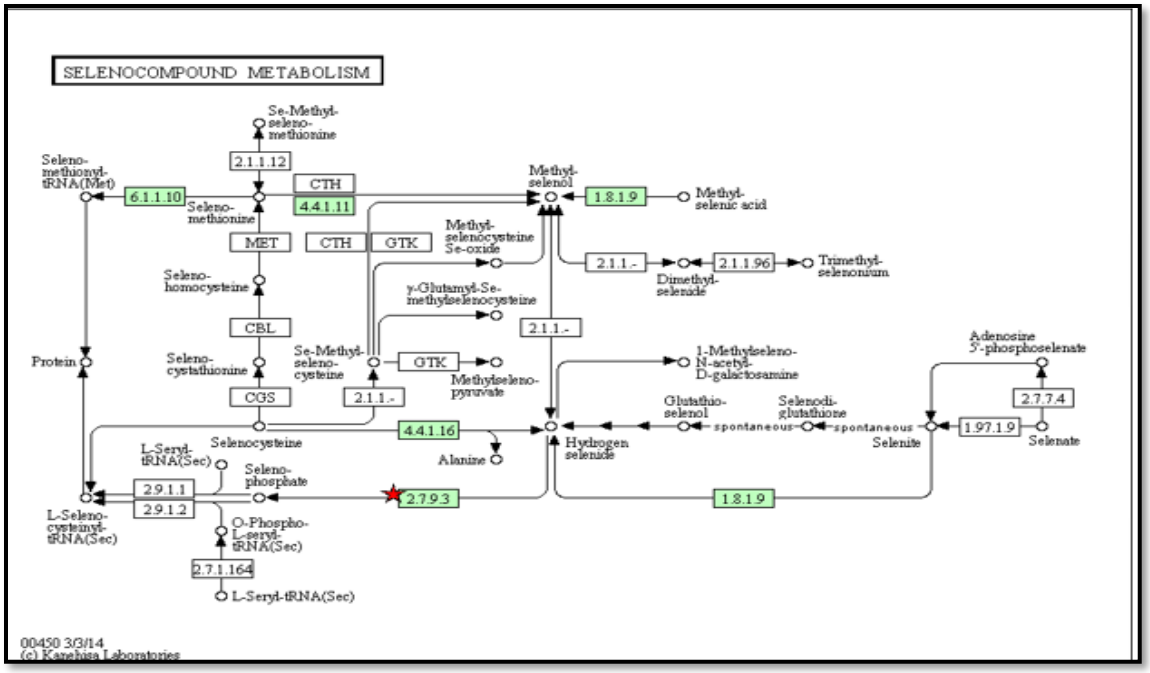

Figure 7g: Selenocompound Metabolism (pathway enriched among conditional essential *P. gingivalis* genes in GAM-CSE). KEGG pathways (www.genome.jp/kegg) are presented with the proteins catalyzing each step shown by their enzyme commission number and substrates/products. Green shading indicates the presence of homologs in *P. gingivalis*. The red star denotes essential genes. The PGN assignments of the essential metabolic genes are provided in Table 11.

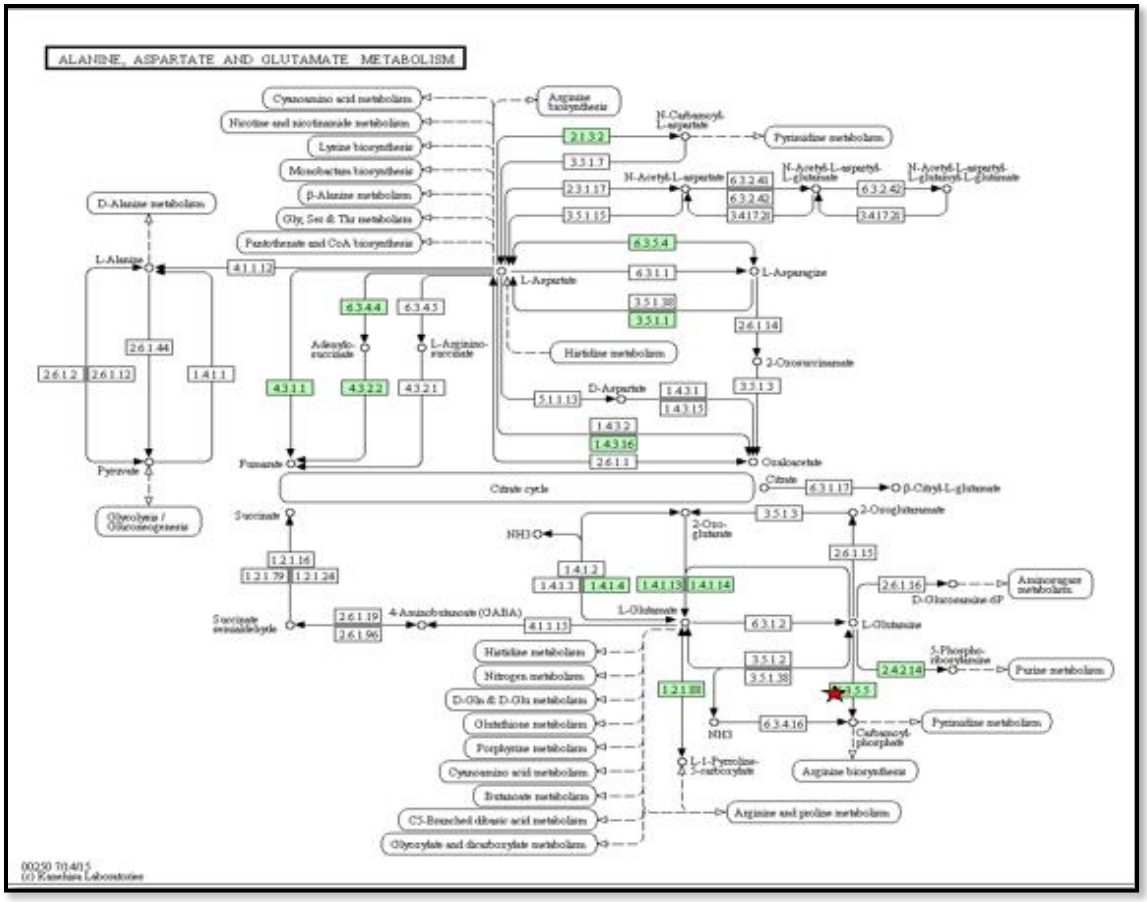

Figure 7h: Alanine, Aspartate and Glutamate Metabolism (pathway enriched among conditional essential *P. gingivalis* genes in GAM-CSE). KEGG pathways (www.genome.jp/kegg) are presented with the proteins catalyzing each step shown by their enzyme commission number and substrates/products. Green shading indicates the presence of homologs in *P. gingivalis*. The red star denotes essential genes. The PGN assignments of the essential metabolic genes are provided in Table 11.

Dpp	PGN_0002	PGN_0003	PGN_0009	PGN_0010	PGN_0013	PGN_0014	PGN_0016	PGN_0017
PGN_0025	PGN_0033	PGN_0064	PGN_0065	PGN_0068	PGN_0070	PGN_0073	PGN_0075	PGN_0076
PGN_0079	PGN_0081	PGN_0082	PGN_0085	PGN_0088	PGN_0090	PGN_0109	PGN_0120	PGN_0121
PGN_0125	PGN_0126	PGN_0129	PGN_0139	PGN_0140	PGN_0141	PGN_0148	PGN_0154	PGN_0156
PGN_0159	PGN_0168	PGN_0174	PGN_0199	PGN_0200	PGN_0221	PGN_0223	PGN_0224	PGN_0229
PGN_0231	PGN_0270	PGN_0271	PGN_0288	PGN_0290	PGN_0291	PGN_0295	PGN_0297	PGN_0302
PGN_0303	PGN_0306	PGN_0314	PGN_0317	PGN_0323	PGN_0335	PGN_0344	PGN_0345	PGN_0351
PGN_0373	PGN_0375	PGN_0388	PGN_0391	PGN_0405	PGN_0407	PGN_0415	PGN_0416	PGN_0431
PGN_0447	PGN_0448	PGN_0450	PGN_0460	PGN_0464	PGN_0483	PGN_0491	PGN_0519	PGN_0533
PGN_0534	PGN_0557	PGN_0561	PGN_0563	PGN_0564	PGN_0566	PGN_0581	PGN_0584	PGN_0590
PGN_0591	PGN_0604	PGN_0607	PGN_0637	PGN_0649	PGN_0650	PGN_0653	PGN_0656	PGN_0658
PGN_0672	PGN_0673	PGN_0675	PGN_0707	PGN_0709	PGN_0711	PGN_0714	PGN_0718	PGN_0719
PGN_0720	PGN_0741	PGN_0748	PGN_0754	PGN_0770	PGN_0771	PGN_0779	PGN_0780	PGN_0788
PGN_0794	PGN_0795	PGN_0827	PGN_0836	PGN_0852	PGN_0857	PGN_0862	PGN_0863	PGN_0872
PGN_0874	PGN_0876	PGN_0880	PGN_0890	PGN_0898	PGN_0900	PGN_0904	PGN_0917	PGN_0922
PGN_0929	PGN_0930	PGN_0972	PGN_0980	PGN_1009	PGN_1014	PGN_1016	PGN_1035	PGN_1036
PGN_1037	PGN_1038	PGN_1039	PGN_1049	PGN_1061	PGN_1073	PGN_1081	PGN_1094	PGN_1100
PGN_1107	PGN_1112	PGN_1116	PGN_1120	PGN_1121	PGN_1122	PGN_1127	PGN_1158	PGN_1159
PGN_1176	PGN_1199	PGN_1200	PGN_1201	PGN_1214	PGN_1227	PGN_1246	PGN_1268	PGN_1269
PGN_1270	PGN_1309	PGN_1310	PGN_1313	PGN_1317	PGN_1321	PGN_1329	PGN_1334	PGN_1335
PGN_1339	PGN_1347	PGN_1348	PGN_1349	PGN_1362	PGN_1366	PGN_1369	PGN_1370	PGN_1374

Table 10: Gene list of the conditional essential genes identified in GAM-CSE model (n=258).

Pathways	Number of genes identified	Genes identified
Pyrimidine metabolism	6	PGN_0375 PGN_1158 PGN_1412 PGN_1443 PGN_1444 PGN_1999
Nicotinate and nicotinamide metabolism	7	PGN_0533 PGN_0534 PGN_0714 PGN_1120 PGN_1121 PGN_1122 PGN_1412
Purine metabolism	1	PGN_1412
Pentose phosphate metabolism	1	PGN_1941
Nitrogen metabolism	1	PGN_1746
Seleno-compound metabolism	1	PGN_1618
Alanine aspartate and glutamate metabolism	2	PGN_1443 PGN_1444
Cysteine and methionine metabolism	3	PGN_1475 PGN_1618 S-ribosyl homocysteine lyase
Mismatch repair	1	PGN_1581
Aminoacyl-tRNA biosynthesis	1	PGN_1646
ABC transporters	2	PGN_0707 PGN_0857

Table 11: Summary of the genes identified in different pathways using KEGG analysis.

UF	gagcgaattCCATCAGCATCCCTGTAGTG
UR	CCAAACTCGCTCTCCAGCTAAAcagctgatag
DF	gatatctagaCATTGGCACAGCCCCGATTCC
DR	CCAAACTCGCTCTCCAGCTAAAcagctgatag

Table 12: Primers used for mutant construction.

4.4. Discussion

P. gingivalis, a Gram-negative periodontal pathogen, must overcome the environmental stress in the periodontal pocket for its survival and growth. Bacterial adaptation to environmental cues involves elaborate regulation of several mechanisms [124]. We were interested in identifying conditionally essential genes for *P. gingivalis* survival in GAM-CSE, using the generated *P. gingivalis* ATCC 33277 Tn-seq library. We identified 258 essential genes for *P. gingivalis* survival in GAM-CSE. These identified genes account for 8% of genes in *P. gingivalis* ATCC 33277 genome.

PGN_0714 encodes a protein, probable pyrazinamidase/nicotinamidase, involved in the nicotinate and nicotinamide metabolism – redox reaction, thus protecting the bacteria from the oxidative stress induced by cigarette smoke. PGN_1524 encodes a tyrosine kinase which is required for secretion of extracellular polysaccharide and biofilm formation with *S. gordonii* [350]. Bacterial kinases have also been shown to be involved in stress responses, antibiotic resistance, and DNA metabolism [351-353]. PGN_0088 (*sinR*), encodes putative transcriptional regulator *sinR* involved in sequence-specific DNA binding. *sinR* ortholog PGN_0088 inhibits polysaccharide synthesis and affects the structure of EPS in *P. gingivalis* ATCC 33277 biofilms [354]. Further, *sinR* mutation resulted in increased production of exopolysaccharide, leading to increased resistance to physical disruption [354]. PGN_1444 encodes a protein involved in pyrimidine biosynthetic pathway and glutamine and arginine metabolic processes. PGN_1200 encodes a protein involved in DNA replication and repair. PGN_0491 (Ltp1) encodes phosphotyrosine protein phosphatase probable phosphotyrosine protein phosphatase, which regulates the expression of *luxS* and exopolysaccharide synthesis, which play a significant role in biofilm formation[355]. PGN_0287 (*mfa1*), encodes minor fimbrium subunit *mfa1*. *mfa1* is involved in bacterial attachment to the host cell surface and other bacteria. Thus, *mfa1*

mediates biofilm formation [356-358]. The competition assay results of the genes described so far provide insight into the gene fitness of genes of interest in the GAM-CSE model, to promote *P. gingivalis* growth in media conditioned with cigarette smoke, which may have relevance in infection and colonization *in vivo*.

Also in the GAM-CSE model, genes involved in carbohydrate and amino acid metabolism; ABC transporters were identified as essential genes. The major habitat of *P. gingivalis* is the subgingival sulcus in the oral cavity. It relies on the fermentation of amino acids for energy production when sugar availability is low [359]. Also, *P. gingivalis* serves as a secondary colonizer of dental plaque, often adhering to primary colonizers such as *S. gordonii* and *Prevotella intermedia.* ABC transporter genes are believed to provide resistance to *P. gingivalis* from potentially harmful chemicals in cigarette smoke [180]. Several genes encoding nucleotide, energy, cofactors and vitamin metabolism; translation, replication, and repair were also identified as essential genes in GAM-CSE model. Replication and repair genes protect bacteria from damage as a result of the oxidative stress induced by cigarette smoke.

Future studies using site-directed mutagenesis of top genes according to fold change in the GAM-CSE model, and complementation assays of the mutants, can be done to further confirm and identify the genes unique to GAM-CSE model. Data generated would provide a better understanding of specific genes, as well as developing novel variants of genes of interest. In summary, despite reduced clinical inflammation, smokers are more prone to bacterial infection and to develop periodontitis. Our experimental results identified several genes essential for *P. gingivalis* growth and survival when exposed to cigarette smoke. Similar analyses can be carried out in other *P. gingivalis* strains, which in turn may provide insight into the common strategies among many bacteria. Apart from smoking, the TnSeq library can be tested under other conditions relevant to periodontal

diseases, including abscess, biofilm, and TIGK invasion models. In the long-term similar analyses, under different conditions and on other bacterial pathogens, to distinguish between inherently essential and conditionally essential genes, may reveal additional targets for new therapeutics.

CHAPTER 5

SUMMARY

Periodontal diseases are multifactorial. In this study, we focused on the relationship between gestational diabetes mellitus and oral infection with periodontopathogens, and the influence of each on systemic inflammation in pregnant women. Also, we focused on identification of *P. gingivalis* ATCC 33277 inherently essential genes and conditional essential genes in media conditioned with cigarette smoke.

Gestational diabetes mellitus is a significant risk factor for periodontal diseases. Pregnancy gingivitis is associated with a hormonal imbalance in the pregnant women. As stated earlier, in this study we are interested in the microbiological aspect of gingivitis in pregnant women. It has been found that the gingivitis during pregnancy was associated with the increased oral infection with *P. gingivalis*, *F. alocis*, and *T. denticola* and combinations of the three. Gestational diabetes mellitus was also associated with increased infection with individual and multiple periodontopathogens. An interesting finding observed in our study is that it is the gingivitis, rather than GDM, that is the driving force for the increased systemic inflammation in pregnant women.

We successfully generated a *P. gingivalis* ATCC 33277 transposon library covering a wide range of the genome which enabled us to identify the inherently essential genes in *P. gingivalis* ATCC 33277 strain. Further, we compared the generated library with the existing library (Klein *et al*., 2012) in order to identify the common inherently essential genes. We entered the identified inherently essential genes in DEG database, which has only 36 bacterial essential genes apart from *P. gingivalis*. As the database grows, we can compare the obtained inherently essential gene list to essential gene list of other bacteria. This comparison, in turn, will help us to identify the species specific essential genes and also

essential genes common in many bacterial species. These identified genes might serve as new therapeutic targets to combat *P. gingivalis* specific infection or to control several infections at the same time. Further, the *P. gingivalis* ATCC 33277 transposon library can be tested under variable conditions such as cigarette smoke, invasion model and mouse models to identify the conditional essential genes.

Cigarette smoke is an important environmental modifiable risk factor for periodontal diseases. Despite reduced clinical signs of periodontal inflammation in smokers, cigarette smoke is known to increase periodontal pathogen infection. The exact reason for the increased periodontal pathogen infection is unclear. For periodontal pathogens, such as *P. gingivalis* to induce or exacerbate periodontal diseases in smokers, they must first be able to survive the highly complex composite toxic insult represented by cigarette smoke. While *P. gingivalis* is resistant to very high doses of cigarette smoke and tobacco constituents, the survival mechanisms are entirely unknown. We identified conditional essential genes for *P. gingivalis* survival in GAM-CSE, which are involved in several metabolic pathways, which enable *P. gingivalis* to combat the stress induced by cigarette smoke. Apart from the genes involved in metabolic processes, several genes involved in *P. gingivalis* attachment to host tissues and other bacterial species were also identified in GAM-CSE model. The genes identified so far are from several studies that have shown increased homotypic and heterotypic biofilm formation by *P. gingivalis* under the influence of cigarette smoke [157, 197, 360]. These findings support the idea of increased periodontal pathogen infection in smokers as a result of the dysbiotic influence of *P. gingivalis*.

From the above studies, we can state that oral pathogens drive the systemic inflammation in pregnant women and, also that *P. gingivalis* adapts to environmental cues. It can also be inferred that these adaptations are essential to enabling its survival. Further, adaptive

mechanisms may also promote bacterial interaction with other oral pathogens, which in turn might lead to periodontal destruction.

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APPENDIX A

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PUBLICATIONS

October 2015 Hutcherson, J. A, **Gogeneni, H**, Yoder-Himes, D, Hendrickson, E. L, Hackett, M, Whiteley, M, Lamont, R. J, Scott, D.

A Comparison of inherently essential genes of *Porphyromonas gingivalis* identified in two transposon-sequencing libraries.

Molecular Oral Microbiology, DOI: 10.1111/omi.12135

June 2015 **Gogeneni, Himabindu**, Buduneli, Nurcan, Ceyhan-Öztürk, Banu, Gümüş, Pınar, Akcali, Aliye, Zeller, Iris, Renaud, Diane E, Scott,David A, Özçaka, Özgün

Increased infection with key periodontal pathogens during gestational diabetes mellitus

Journal of Clinical Periodontology, DOI: 10.1111/jcpe.12418